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NOK mediated mitogenic signaling is altered by P203L and V395I mutations

Sheng-Qi Hou[1], Li Liu[1]

[1]Department of Microbiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Union Medical College, Beijing 100005, China

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1. ABSTRACT

The novel oncogene with kinase-domain (NOK), is an atypical receptor protein tyrosine kinase with potent oncogenic potential. In the current study, we generated two point mutations (P203L and V395I) on NOK gene. NOK(P203L) is identical to serine/threonine/tyrosine kinase 1 (STYK1), the aliases of NOK, while the V395I mutation was recovered from human glioblastoma. Both mutations did not impair NOK kinase activities, but V395I inhibited NOK autophosphorylation. Although with overall inhibition, both STYK1 and V395I affected the activities of extracellular regulated protein kinase (ERK), Akt and signal transducer and activator of transcription (STAT) differently in HEK293T cells versus HeLa and BaF3 stable cells The proliferation potentials for both STYK1 and V395I were significantly inhibited. Single mutation at either site was sufficient to abolish the IL-3 independent growth and the anchor-independent growth of of BaF3 stable cells. Overall, our data indicates that both P203 and V395 residues on NOK are important for NOK mediated mitogenic signaling, and the substitutions of P203L and V395I may selectively affect certain mitogenic signaling cascades in a tissue specific manner.

2. INTRODUCTION

The receptor protein tyrosine kinases (RPTKs) are enzymatic proteins that mediate a variety of cellular activities including cell proliferation, differentiation, cell cycle control, embryogenesis, angiogenesis, and metabolism (1-3). Due to their importance in normal cell communications, their activities are usually tightly regulated. However, when the normal activity of RPTK is disturbed, it may bring severe disorders like diseases or even cancers (4-8). In a classical point of view on RPTK activation in general, ligand usually first recognizes its specific extracellular domain, and subsequently triggers the formation of homo- or hetero-dimmer (1, 9). The intracellular domain of one of the dimmer will function as the kinase to phosphorylate its counterpart, resulting in the mutual activation of the receptor complex (1, 9). The phosphorylated sites of the dimmer often serve as the docking sites for the subsequent recruitment of the key effector and/or adaptor proteins which often contain Src homology2 (SH2) and phosphotyrosine binding (PTB) domains to the receptor for the activation of diverse downstream signaling cascades (10).

NOK (novel oncogene with kinase-domain) is a lately discovered RPTK and may stand out as a unique

RPTK family (11, 12). Previous studies have showed that NOK is a potent oncogene with strong tumorigenic and metastasistic properties (11, 13). Systematic analysis on human cancer genomes identified V395I mutation as a somatic mutation associated with human glioblastma (4). Compared to NOK, STYK1 has only one amino acid substitution at the site of amimo acid 203 (P203L), whose gene product was first cloned from human fetal brain cDNA library by Ye *et al* (14). In this study, we generated these two mutants based on NOK gene and used either transiently transfected or stably expressed systems to dissect their functional differences with respect to kinase properties and NOK mediated signaling cascades. We found that the overall proliferation potentials of both STYK1 and V395I were significantly reduced as compared with NOK, while both mutations did not affect their respective kinase activities.

3. MATERIAL AND METHODS

3.1. Plasmid construction and site-directed mutagenesis

The full length of NOK (Genbank accession number: KP729000) with the influenza hemagglutin (HA) epitope tag was sub-cloned into the XbaI and NotI (Takara, Dalian, Liaoning, China) sites of pCDH-CMV-MCS-EF1-CD511B (SBI, Mountain view, CA) to form pCDH-CMV-MCS-EF1-CD511B-NOK-HA plasmid, and sub-cloned into the HindIII and XbaI sites of pCDNA3.0 to form pCDNA3.0-NOK plasmid. The mutant constructs of pCDH-CMV-MCS-EF1-CD511B-STYK1-HA and pCDH-CMV-MCS-EF1-CD511B-NOK(V395I)-HA, as well as pCDNA3.0-STYK1-HA and pCDNA3.0-NOK(V395I)-HA were generated by Takara MutantBEST Kit (Catalog number D401; Dalian, China) following the manufacturer`s instruction. The sense primers for these two mutant constructions are 5'-GGGGACCTGCTCAGCTTTCTC-3' and 5'-GTGTTACAAATACCAGAGTTGGTGG-3', respectively. The antisense primers for these two mutants are 5'-CTGGGCCACATCCTCCAACAC-3' and 5'-AGCCTCGTCATCTGCAGTTTTA-3', respectively. The PCR reaction mixture was amplified by 30 cycles at 94^{o} C for 50 seconds, 55^{o} C for 50 seconds, and 72^{o} C for 120 seconds. All plasmids were constructed using standard PCR and cloning strategies. The products were first sub-cloned into PMD-18T-Simple vector and then cloned into pCDH-CMV-MCS-EF1-CD511B. All plasmids were confirmed by double digestion and subsequent sequencing analysis.

3.2. Cell culture, transient transfection and antibodies

Human embryonic kidney cell line HEK293T, human cervix carcinoma cell line HeLa, murine myelomonocytic leukaemia cell line WEHI-3B, and murine pro-B cells BaF3 were obtained from the Cell Culture Center of Basic Institute of Medical Sciences, Chinese Academy of Medical Sciences. HEK293T and HeLa cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. BaF3 and WEHI-3B cells were cultured in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. At about 80% confluency, HEK293T and HeLa cells were transiently transfected with plasmid DNAs by transfection reagent VigoFect (Vigorous, Inc., Beijing, China) by following its manual instruction. Antibodies used were: anti-HA (1/5000, Proteintech), anti-Erk and anti-p-Erk(1/1000, Santa Cruz Biotechnology), anti-Akt and anti-p-Akt (1/1000, Santa Cruz Biotechnology), anti-STAT1 and anti-p-STAT1 (1/1000, Bioworld Biotechnology), anti-STAT 3 and anti-p-STAT3 (1/1000, Santa Cruz Biotechnology), anti-STAT5 and anti-p-STAT5 (1/1000, Bioworld Biotechnology) and anti-β-actin (1/1000, Zhong Shan Jin Qiao Biotechnology, Beijing, China).

3.3. Generation of BaF3 stable cells

HEK293T/N packaging cells were transiently co-transfected with the lentiviral vectors (pCMV-VSV-G, pMD2.G and pRSV-Rev) plus the pCDH-CMV-MCS-EF1-CD511B containing the gene of interest by VigoFect. After 48 h transfection, the lentivirus-containing medium was collected and supplemented with 8mg ml^{-1} Polybrene (Sigma). Then the wild type BaF3 cells were incubated with the harvested cell supernatant that contains the reconstituted lentivirus for 12 hrs. Stable cell clones were identified based on the expression of lentiviral EF1-driven GFP by flow cytometric analysis, RT-PCR and western blot analysis.

3.4. Western blot analysis

Cells were collected and washed with cold 1xPBS and lysed in a reaction buffer containing 20 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5μg ml leupeptin (pH7.5). Equal amount of proteins in the cell lysates were first loaded and separated on to 10% SDS-PAGE. Then the SDS-PAGE gel was electrotransferred to a nitrocellulose membrane (GE Health Care, USA) at 300mA for 2.5 hours. After blocking with 5% slim milk for 1 hour at room temperature, the transferred membrane was first probed with primary antibodies at 4°C overnight. Then the membrane was probed with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Finally, the reaction products were developed by enhanced chemiluminescence kit (Santa Cruz, CA).

3.5. Cell proliferation assay and anchor independent growth assay

For cell proliferation assay, the transfected HEK293T and HeLa cells were seeded onto 96-well microtiter plates at a density of 1×10^4 per well and sub-cultured for the indicated time periods. Proliferation rates were assessed by the color change of WST-8 using a cell

counting kit-8 (cell counting kit-8, Dojindo, Japan) under standard protocol. For anchor independent growth assay, about 1×10^4 stable BaF3 cells (BaF3-EV, BaF3-NOK, BaF3-STYKI, BaF3-NOK(V395I)) were re-suspended into 2 ml of 0.4.% agar in 1x RPMI1640 supplemented with 10%FBS. The cell suspension mixture was then put onto a bottom layer which was a 2 ml of 0.7.% agar made in 1x RPMI1640 supplemented with 10%FBS. After the top layer became solid, about 1.5 ml of 1x RPMI1640 plus 10% FBS was added onto each top layer of the 6-well plate and was continuously incubated at 37^o C in the presence of 5% CO_2 for 2 weeks. The presence of colonies was assessed and the number of colonies whose diameters were >0.2mm in each group was quantified.

3.6. Immunoprecipitation

HEK293T cells were transiently transfected with the indicated plasmids by transfection reagent VigoFect following the standard manufacture's protocol. At 48h post-transfection, cells were collected and washed with cold 1xPBS and lysed in a lysis buffer (50mM Tris, at pH7.4., 150mM NaCl, 10% glycerol, 1mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Thermo). The clarified lysates were incubated with anti-HA antibody coupled beads at 4^o C overnight. The HA beads (Thermo) were then spun down at 8000 × g for 30 seconds and washed five times using the lysis buffer following standard protocol. The immunoprecipitated proteins attaching to the beads were boiled in SDS-PAGE loading buffer containing 1% SDS, 5mM dithiothreitol, 1mM sodium vanadate and 50mM Tris at pH7.4 for 10 minutes. Then the samples were resolved on 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk, the membranes were probed with corresponding primary antibody followed by HRP-conjugated secondary antibody. Finally, the reaction products were developed by enhanced chemiluminescence kit (Santa Cruz).

3.7. Autophosphorylation assay

HEK293T cells were transiently transfected with pCDNA3.0, pCDNA3.0-NOK, pCDNA3.0-STYK1 and pCDNA3.0-V395I using VigoFect. After 48h post-transfection, cells were collected and washed with cold 1× PBS twice. Then cells were collected by 4000rpm centrifugation for 5 minutes, and lysed with RIPA supplemented with protease and phosphatase inhibitors (Thermo) for 30 minutes at 4^{o}C. The supernatant was immunoprecipitated with anti-HA beads at 4^{o}C overnight. Beads were then spun down at 8200 × g for 30 seconds and washed five times using the lysis buffer by following the standard protocol. The immunoprecipitated proteins were then analyzed by western blot. Anti-p-Tyr antibody was used to detect the phosphorylation levels of NOK and its mutant derivatives.

3.8. *In vitro* tyrosine kinase activity assay

HEK293T cells were transiently transfected with pCDNA3.0, pCDNA3.0-NOK, pCDNA3.0-STYK1 and pCDNA3.0-V395I using VigoFect. At 48h posttransfection, cells were collected and washed with cold PBS and lysed in the lysis buffer (same as above) supplemented with protease and phosphatase inhibitors. Total proteins in cell lysates were quantified by BCA protein assay kit (Thermo). Then 5μg total proteins were tested for tyrosine kinase activity. Briefly, 5μg total protein was incubated for 30 min at room temperature with substrates peptides which were bound to streptavidin presented well-strips in 50μl of kinase reaction buffer (100mM Tris-HCl, pH7.4, 50mM $MgCl_2$, 5mM $MnCl_2$, 5mM dithiothreitol, 1mM ATP, 50mM sodium orthovanadate). At the end of the incubation, stripes were washed five times with 1 × TBS/T. Then each well-stripe was added 100μl of anti-phosphotyrosine, recombinant 4G10TM plus HRP conjugate, and incubated for 20-30 min at room temperature. After washing five times, stripes were further incubated with 75μl mixture of TMB-Substrates for up to 15 minutes. Reaction was stopped by adding 2M sulfuric acid. The absorbance in each well was measured using a spectrophotometric plate reader at a wavelength of 450nm.

4. RESULTS

4.1. NOK and its two mutant derivatives STYK1 and V395I

STYK1 is the aliases of NOK with one amino acid (aa) substitution from praline (P) to leucine (L) at aa203, while the valine (V) to isoleucine (I) substitution at the aa395 was considered to be a somatic mutation for human glioblastoma by cancer genomic study (4). P203L mutation is located at the sub-domain 1 of NOK kinase domain, while the V395I mutation is proximal to the carboxyl terminus of NOK (Figure 1). In this study, two mutant derivatives of NOK which were named NOK(P203L) or STYK1 and NOK(V395I) were generated by standard site mutagenesis. We are interested in understanding whether these two mutation sites could influence NOK biological activity, and whether they attribute to the activations of NOK-mediated mitogenic signaling cascades.

4.2. The effects of STYK1 and V395I on NOK autophosphorylation and kinase activity

The first question that we tried to ask was whether these two mutations could affect the autophosphorylation level of NOK. First, NOK and its mutants STYK1 and V395I were transiently transfected into HEK293T cells. Cell lysates were then prepared and immunoprecipitated with anti-HA beads. Figure 2A shows that the phosphorylated level of STYK1 was similar to NOK, however, the mutant V395I inhibited NOK mediated autophosphorylation. To further determine whether mutation have any effect on kinase activity,

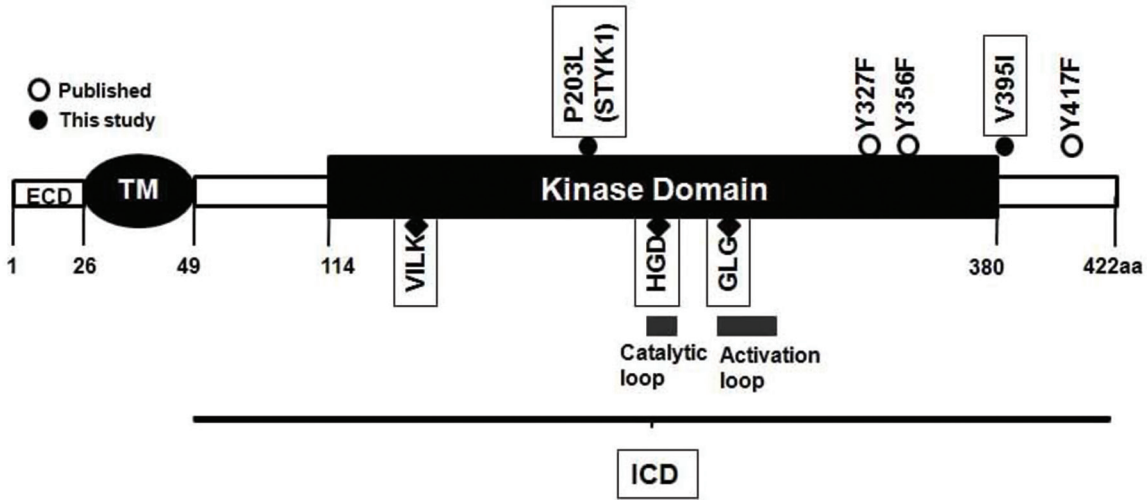

Figure 1. NOK protein structure and its mutant derivatives. Black dots represent the amino acid substitutions carried out in this study, white dots indicate the previously studied NOK mutants, while solid diamonds represent the conserved motifs in NOK kinase domain. The relative catalytic and activation domains are also indicated. ECD: extracellular domain; ICD: intracellular domain; TM: transmembrane domain.

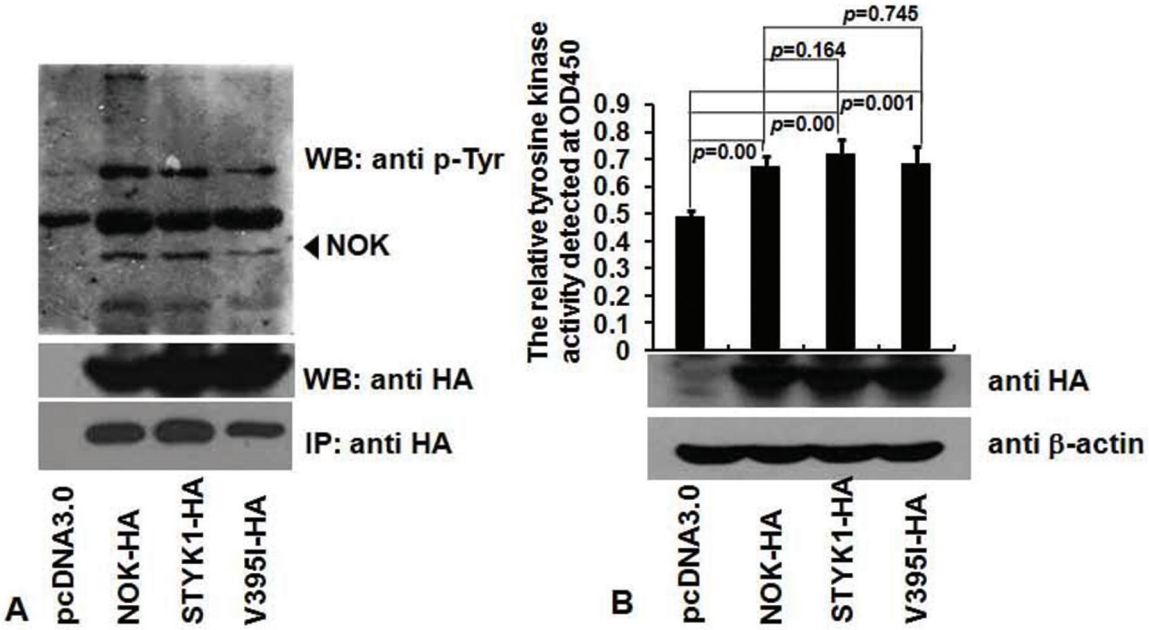

Figure 2. (A) Autophosphorylation activity of NOK wild type and its mutants. The HEK293T cell was transiently transfected with the empty vector (pcDNA3.0), pcDNA3.0-NOK-HA, pcDNA3.0-STYK1-HA and pcDNA3.0-NOK(V395I)-HA. Cell lysates were first immunoprecipitated with anti-HA antibody and then the membranes were probed with anti-p-Tyrosine antibody. The data is the representative of three independent experiments with similar results. (B) Mutations at P203 and V395 sites did not affect the tyrosine kinase activity of NOK. HEK293T cells were transiently transfected with pcDNA3.0, pcDNA3.0-NOK-HA, pcDNA3.0-STYK1-HA and pcDNA3.0-NOK(V395I)-HA. After 48h transfection, about 5μg of the clarified lysates were incubated with poly-peptide substrates to exam the tyrosine kinase activity. The value is represented by the mean ± standard deviation (SD).

we used *in vitro* kinase assay system to evaluate their respective kinase activities. HEK293T were transiently transfected with pcDNA3.0, pcDNA3.0-NOK, pcDNA3.0-STYK1 and pcDNA3.0-NOK(V395I). After 48-h post-transfection, the cell lysates were assayed for kinase activity. Figure 2B shows that NOK, STYK1 and V395I all displayed significantly enhanced tyrosine kinase activity as compared with the negative control. Although V395I mutation inhibited the NOK autophosphorylation, both mutations did not alter the kinase activity of NOK, indicating that the phosphorylation status of NOK may not be the prerequisite for its kinase activation.

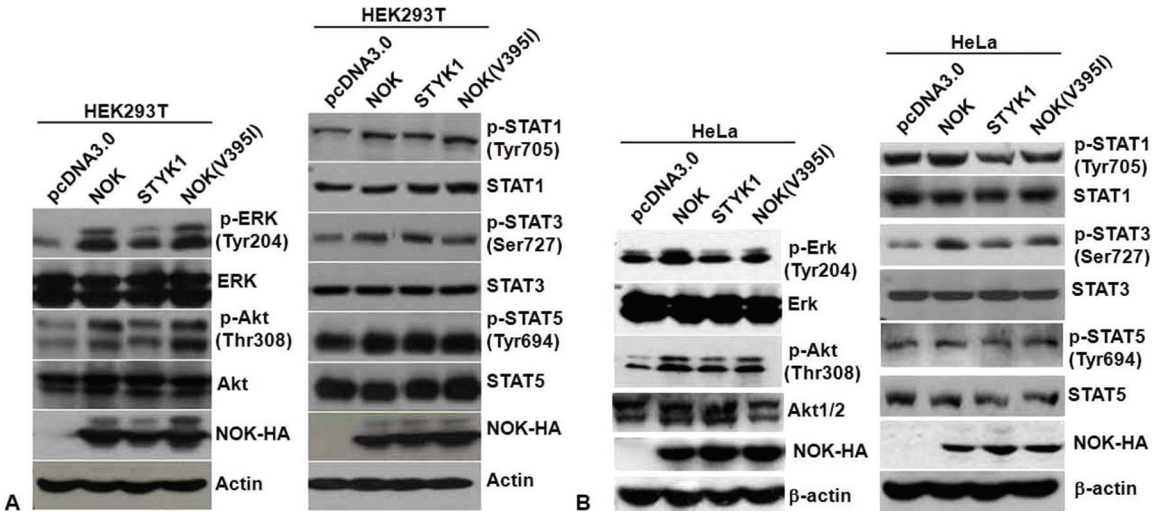

Figure 3. Effects of STYK1 and V395I on NOK mediated signaling transductions in HEK293T and HeLa cells. Both HEK293T (A) and HeLa (B) cells were transiently transfected with pcDNA3.., pcDNA3.0-NOK-HA, pcDNA3.0-STYK1-HA and pcDNA3.0-NOK(V395I)-HA. After 48h transfection, the cell lysates were subjected to western blot analysis. The reaction products were probed by using anti-ERK, anti-phosphorylated ERK (p-ERK) (Tyr204), anti-Akt, anti-phosphorylated Akt (p-Akt) (Thr 308), anti-STAT1, anti-phosphorylated STAT1(p-STAT1) (Tyr705), anti-STAT3, anti-phosphorylated STAT3 (p-STAT3) (Ser727), anti-STAT5 and anti-phosphorylated STAT5 (p-STAT5) (Tyr694). The cells transfected with empty vector (pcDNA3.0) are served as controls.

4.3. The effects of STYK1 and V395I on NOK mediated signaling pathways

Next, we explored the roles of NOK mutant derivatives in NOK mediated signaling transductions. First, we used transient transfection approach to dissect the effects of NOK mutant derivatives on the major mitogenic signaling pathways such as PI3K/Akt, RAS/MAPK and JAK/STAT. Plasmids pcDNA3.0, pcDNA3.0-NOK, pcDNA3.0-STYK1 and pcDNA3.0-NOK(V395I) were transiently transfected into either HEK293T or HeLa cells. After 48h posttranfection, western blot analysis was performed to investigate the effects of STYK1 and V395I on mitogenic signaling pathways. Figure 3A shows that NOK was able to enhance phosphorylation levels of both ERK and Akt in HEK293T cells. However, mutant V395I did not reduce the phosphorylation levels of NOK mediated activations on ERK and Akt, In contrast, STYK1 inhibited the activation of these two signaling molecules in HEK293T cells. Both mutations did not affect the phosphorylated levels of STAT1, 3 and 5 in HEK293T cells except that V395I mutatant could selectively inhibit STAT3 activation (Figure 3A). Differently, in HeLa cells, STYK1 down-regulated the phosphorylation levels of both ERK and Akt, while V395I only inhibited the activation of ERK but not Akt (Figure 3B). In addition, both mutants reduced the phosphorylation levels of STAT1 and STAT3, while the phosphorylation levels of STAT5 were inert to be changed in responding to the over-expressions of NOK and its mutant derivatives in HeLa cells (Figure 3B).

The effects of NOK and its mutant derivatives on NOK mediated signal transductions were also evaluated in BaF3 stable cells. BaF3 stable cells expressing NOK, STYK1 or V395I were established by co-transfecting NOK or its mutant derivatives with lentiviral packaging system into BaF3 cells. BaF3 cell generated by empty lentiviral particles was served as negative control. Figure 4 demonstrates that both STYK1 and V395I mutants not only markedly inhibited STAT1, STAT3 and STAT5 activations but also repressed ERK phosphorylation, while only STYK1 but not V395I significantly decreased the phosphorylation level of Akt (Figure 4). Taken together, the above results indicate that the effects of STYK1 and V395I on NOK-mediated signaling transductions may be different as the tested tissues different.

4.4. The effect of STYK1 and V395I on NOK mediated cell proliferation

To investigate the effects of STYK1 and V395I on NOK mediated cell proliferation, plasmids pcDNA3.0, NOK, STYK1 and V395I were first transiently transfected into HEK293T cells. CCK8 assay was used to measure the cell proliferation potential. A significant reduction in cell proliferation was detected in both STYK1 and V395I after 48h post-transfection (Figure 5A). Similarly, transiently delivering either STYK1 or V395I into HeLa cells also attenuated NOK mediated cell proliferation (Figure 5B). To further consolidate the above results, we assayed the effects of STYK1 and V395I mutants on the proliferation of BaF3 stable cells. After overnight starvation, the cell proliferation potentials of BaF3-NOK and its mutant derivatives were evaluated on day 1, 2 and 3 of post-inoculation in the absence of serum and IL-3. No significant difference was observed among NOK and the two mutant derivatives during the first two days of incubation. However, after three day incubation, the number of

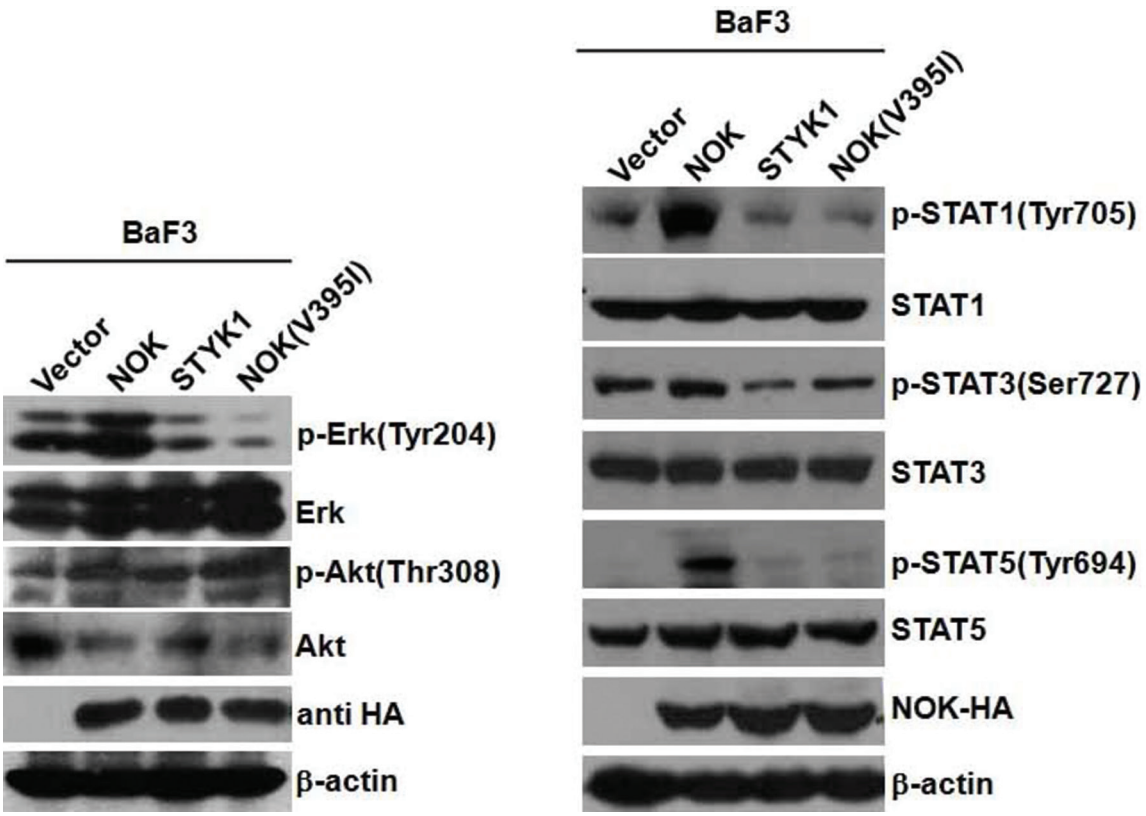

Figure 4. Effects of STYK1 and V395I on NOK mediated signaling transductions in BaF3 stable cells. Cell lysates were prepared from BaF3-CD511B, BaF3-CD511B-NOK-HA, BaF3-CD511B-STYK1-HA and BaF3-CD511B-NOK(V395I)-HA and subjected to western blot analysis. The reaction products were probed by using anti-ERK, anti-phosphorylated ERK (p-ERK) (Tyr204), anti-Akt, anti-phosphorylated Akt (p-Akt) (Thr 308), anti-STAT1, anti-phosphorylated STAT1(p-STAT1) (Tyr705), anti-STAT3, anti-phosphorylated STAT3 (p-STAT3) (Ser727), anti-STAT5 and anti-phosphorylated STAT5 (p-STAT5) (Tyr694). The cells transfected with empty vector (pcDNA3.0) are served as negative controls.

viable cells did not increased for STYK1 group, while the proliferation potential of V395I mutant group markedly decreased (Figure 5C). In contrast, the proliferation potential of NOK group continuously increased for at least 3 days (Figure 5C), indicating that STYK1 and V395I mutants abolished the IL-3 independent growth of BaF3-NOK. Therefore, both mutants have reduced proliferation potentials as compared with the wild type NOK.

4.5. STYK1 and V395I fail to promote anchorage-independent growth of BaF3 stable cells

To directly evaluate the effect of STYK1 and V395I on NOK mediated tumorigenic potential *in vitro*, colony formation assay was performed in soft agar. After starvation for 18 hours, the equal numbers of BaF3-CD511B (negative control), BaF3-NOK, BaF3-STYK1 and BaF3-NOK(V395I) stable cells were seeded onto soft agar made with complete culture medium. Cells were allowed to grow for about 15 days before counting by eyes and under microscopy (Figure 6A and 6B). Quantitative analysis indicates that both STYK1 and V395I could dramatically inhibited NOK induced anchor-independent growth in BaF3 stable cells. Overall, these results indicates that single mutation at either P203 or V395 site could markedly block or delay NOK mediated cell survival and transformation.

5. DISCUSSION

NOK is a lately discovered potent oncogene that could effectively induce tumorigenesis and metastasis in animals. Although the name STYK1/NOK is wildly used in many reports (15-23), the functional differences between STYK1 and NOK have not been systematically investigated. More recently, the valine (V) to isoleucine (I) substitution at the aa395 was identified as a somatic mutation with less driver possibility from human glioma sample (4). In current study, we generated these two single mutations (P203L and V395I) on the oncogene NOK and assayed their effects on NOK mediated signal transductions and proliferation. Interestingly, both point mutations behave little differently and inhibit NOK mediated signaling pathways more severely in cancer cells (HeLa and BaF3-NOK) than in transformed normal cells (HEK293T). More importantly, both mutants

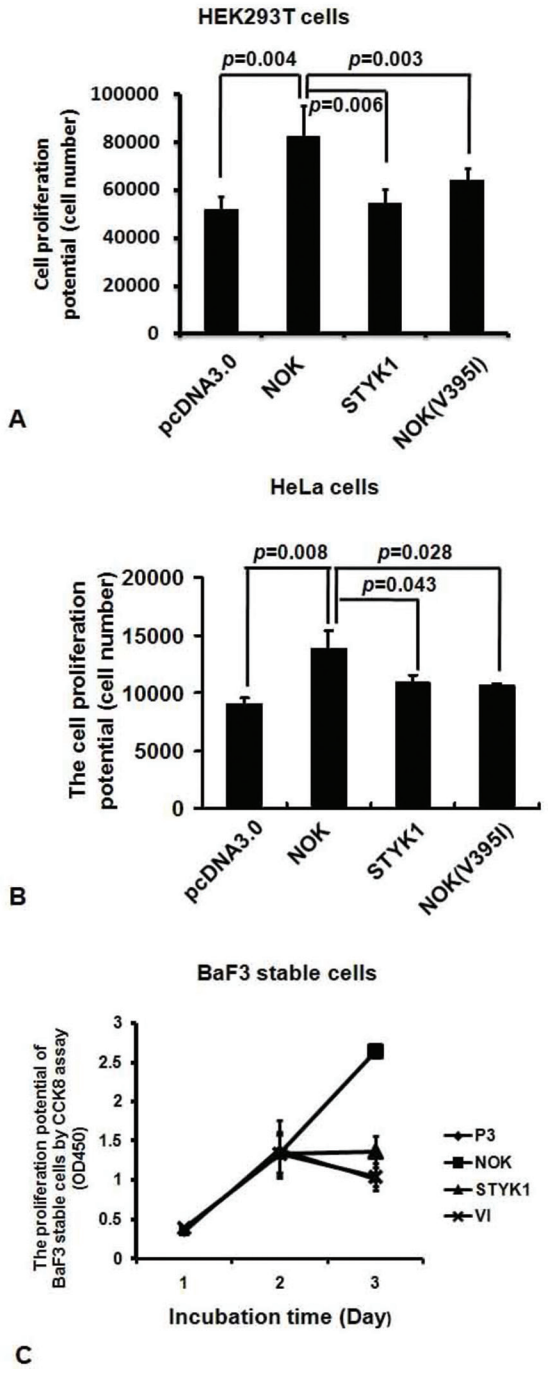

Figure 5. STYK1 and V395I inhibit NOK mediated cell proliferation. HEK293T cells (A) and HeLa cells (B) were transiently transfected with pcDNA3.0, pcDNA3.0-NOK-HA, pcDNA3.0-STYK1-HA and pcDNA3.0-NOK(V395I)-HA. Then, the transfected cells were re-seeded onto 96-well microtiter plate at a density of ~1 $\times 10^4$ per well and cultured for additional 2 days before tested for CCK8. (C) For stable cells, after overnight starvation, about 5×10^4 BaF3 stable cells (Baf3-vector, BaF3-NOK, BaF3-STYK1 and Baf3-NOK(V395I)) were seeded onto a 96-well microtiter plates and cultured for additional 1, 2 or 3 days without serum and IL-3. The cell proliferation rates were assessed by adding cell-counting kit-8 solution into each well that was subsequently measured by spectrophotometer at 450 nm.

(STYK1 and V395I) significantly inhibit the anchorage independent growth of BaF3 stable cells.

Sequence alignment indicates that one amino acid substitution from proline to leucine at aa203 site is found for STYK1, the alias gene of NOK. Although both proline and leucine are nonpolar amino acids, the hydropathy index of proline (1.6) is much lower than leucine (3.8) as based on Kyte and Doolittle's method (24). Thus, proline is a less hydrophobic amino acid. From the structural point of view, proline appears to be a maverick one as compared with leucine and other amino acids. Proline is the only amino acid that is cyclic and its hydrogen atom carries only one rather than two hydrogen atoms. Therefore, proline presents exceptional conformation rigidity that might directly affect the secondary structure of the protein round the proline site. Differently, leucine residue is linear and opened amino acid whose side chain is more flexible than proline. Indeed, growing reports indicate that mutual amino acid substitution between proline and leucine often results in the alteration of the protein integrity that then subsequently affects its functional properties. For example, leucine-to-proline substitution at the aa115 site of MEK (mitogen activated protein kinase kinase) resists to the inhibitory effect of MEK inhibitor PD184352 (25), while proline-to-leucine substitution at the aa850 site of inositol-requiring enzyme 1α completely inhibits its kinase and RNase properties (26). Differently, proline-to-leucine substitution at the aa45 of Cdk2 inhibits its cyclin association but does not alter its kinase activity (27). The role of P203L mutant (STYK1) in NOK is similar to that of P45L mutation in Cdk2. The P203L mutation also does not affect NOK kinase activity but can inhibit the majority of NOK mediated mitogenic signaling pathways in cancer cell lines.

Valine395-to-isoleucine (V395I) substitution in STYK1 was identified as a somatic mutation associated with human glioma (4). Both valine and isoleucnine are hydrophobic amino acids that are supposed to be folded interiorly. Valine is a shorter form of isoleucine that lacks a methal group at its side chain. Therefore, the substitution from valine to isoleucine might alter the protein folding in certain way. Valine395 residue is at the 3' outside of the kinase domain and is proximal to the carboxyl terminus of NOK protein. Tyrosine residues located at the carboxyl terminus of tyrosine kinase are usually crucial for its kinase activity, autophosphorylation and/or signaling functions (28-31). V395I substitution on NOK did not change its kinase activity but inhibited NOK autophosphorylation, transformation potential as well as its downstream signaling. In contrast, study also indicates that valine-to-isoleucine substitution in kinase protein can increase its transforming capacity (32, 33). For example, V157I substitution in the insertionally activated chicken erbB (IA c-erbB) gene can increase its kinase activity and transformation potential in a tissue specific manner (32). Although V395I mutation has reduced autophosporylation

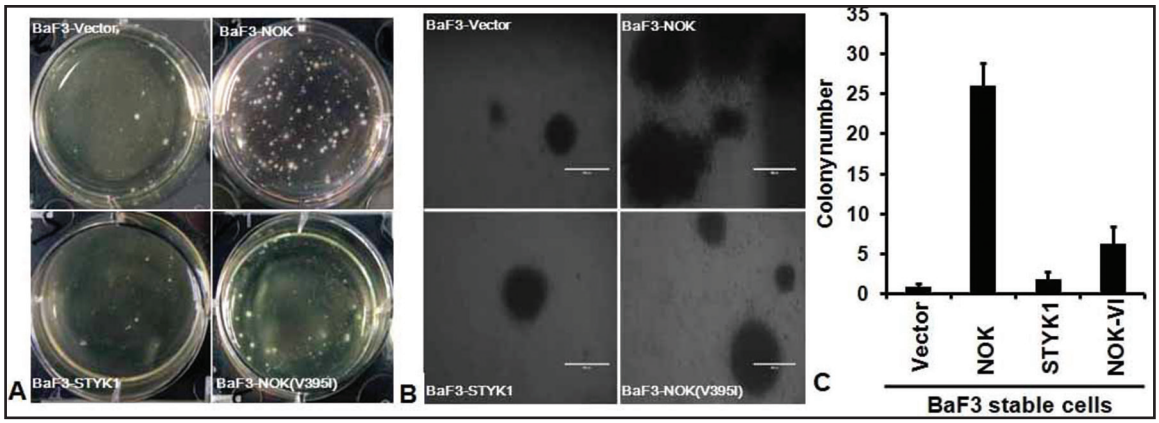

Figure 6. STYK1 and V395I impede NOK mediated anchorage-independent growth. About 5 x 10^4 BaF3 stable cells (BaF3-vector, BaF3-NOK, BaF3-STYK1 and BaF3-NOK(V395I)) were seed in soft agar plates supplemented with completed medium and cultured for about 10 days. Then, the colony formations were observed by eyes (A) and under microscopy (B). Colony diameters >0.2mm were counted as positive. The quantitative analysis was also performed by calculating the mean ± SD for each stable cell line (C).

and presents inhibitory role in the activations of downstream signaling transductions, the"driver" effect of this mutation on neuro cells is still unknown. Therefore, the transformation potential of NOK(V395I) mutation in glia cells is still needed to be evaluated.

Current data demonstrates the functional differences among NOK, STYK1and V395I in both transient transfection and stable expression systems. However, the detailed mechanisms accounted for these differences are still unknown. Recent elegant study by Shan *et al*. shows that the N lobe dimerization interface of EGFR is usually disordered, mutation mediated EGFR dimerization and/or autophosphorylation can suppress this disorder and in the same time increases its kinase activity and transforming ability (34). STYK1 and V395I apparently belong to inhibitory mutations but not driver or passenger mutations. Combining with Shan's report and available data, we speculate that the mechanisms responsible for P203L and V395I mediated inhibition might be different. It might be true that P203L mutation may induce the disorder of N lobe of NOK, while V395I mutation prevents the docking of adaptor protein by interfering with the autophosphorylations of the tyrosine residues at the C terminus of NOK. Although V395I mutation in STYK1 has been defined as a somatic mutation associated with human glioma, the nature of the mutual substitution between proline and isoleucine at aa203 of NOK and STYK1 is still needed to be further investigated. Systematic and large scale sequencing analysis on clinical cancer samples should be performed to clarify this issue in more details in the near future.

6. ACKNOWLEDGEMENTS

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Abbreviation: novel oncogene with kinase-domain (NOK); serine/threonine/tyrosine kinase 1 (STYK1); receptor protein tyrosine kinase (RPTK); Src homology2 (SH2); phosphortyrosine binding (PTB); amino acid (aa); extracellular regulated protein kinase (ERK); Akt (protein kinase B, PKB); signal transducer and activator of transcription (STAT);

praline (P); leucine (L); valine (V); isoleucine (I); insertionally activated chicken erbB (IA c-erbB)

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Send correspondence to: Li Liu, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Uion Medical College, Beijing 100005, China, Tel: 86 10 69156454, Fax: 86 10 65233768, E-mail: lliu@pumc.edu.cn