ANTICANCER RESEARCH *36*: 2733-2744 (2016)

Novel Tumor-specific Mutations in Receptor Tyrosine Kinase Subdomain IX Significantly Reduce Extracellular Signal-regulated Kinase Activity

MASAKUNI SERIZAWA[1], MASATOSHI KUSUHARA[1,2], SUMIKO OHNAMI[3], TAKESHI NAGASHIMA[3,4], YUJI SHIMODA[3,4], KEIICHI OHSHIMA[5], TOHRU MOCHIZUKI[5], KENICHI URAKAMI[3] and KEN YAMAGUCHI[6]

[1]*Drug Discovery and Development Division,* [2]*Region Resources Division,* [3]*Cancer Diagnostics Division, and* [5]*Medical Genetics Division, Shizuoka Cancer Center Research Institute, Shizuoka, Japan;*
[4]*SRL, Inc., Tokyo, Japan;*
[6]*Shizuoka Cancer Center Hospital and Research Institute, Shizuoka, Japan*

Abstract. *Background/Aim: The identification of additional therapeutic targets by clinical molecular profiling is necessary to expand the range of molecular-targeted cancer therapeutics. This study aimed to identify novel functional tumor-specific single nucleotide variants (SNVs) in the kinase domain of receptor tyrosine kinases (RTKs), from whole-exome sequencing (WES) data. Materials and Methods: SNVs were selected from WES data of multiple cancer types using both cancer-related databases and the index reflecting molecular evolution. Immunoblotting and luciferase assay were performed to assess the function of selected SNVs. Results: Among the seven selected SNVs, two, namely neurotrophic receptor tyrosine kinase 1 (NTRK1) V710A and fms related tyrosine kinase 3 (FLT3) K868N, detected in kinase subdomain IX, were investigated. These SNVs inhibited the autophosphorylation of the respective RTKs, thereby reducing the activity of extracellular signal-regulated kinases. Conclusion: RTK subdomain IX is a promising target for the molecular design of kinase inhibitors.*

Recent progress in cancer genomics has revealed somatic genetic alterations, including mutations, copy-number variations and fusions, responsible for cancer progression (1-3). These findings have led to development of molecular-targeted cancer therapeutics against tumors with oncogenic genetic alterations (4, 5). However, only a handful of patients with cancer actually benefit from effective molecular-targeted cancer therapeutics. In order to expand the range of molecular-targeted cancer therapeutics, the identification of additional therapeutic targets through molecular profiling of each patient with cancer is urgently needed (6). Therefore, the Shizuoka Cancer Center launched Project HOPE (High-tech Omics-based Patient Evaluation), that is the first prospective molecular profiling study across multiple types of cancer in Japan, in January 2014 in order to identify patient-specific molecular signatures *via* multi-omics analysis (7).

Meanwhile, the massive increase in studies of cancer genome sequencing has resulted in the accumulation of functionally unknown tumor-specific mutations for which no molecular or clinical study has been performed to determine their roles in the biological and clinical behavior of cancer. The functional characterization of these unknown mutations should facilitate the identification of novel therapeutic targets. Because many genetic alterations in receptor tyrosine kinases (RTKs) have been identified in multiple types of cancer as oncogenic drivers, that eventually activate downstream signaling cascades relevant to cancer-cell survival, mutations detected in RTKs should be considered first in the selection of candidate mutations from massive datasets containing novel tumor-specific mutations which are subjected to further functional evaluation (3, 8-10). Moreover, most mutations identified in RTKs, functioning as oncogenic drivers or relevant to sensitivity to molecular-targeted therapeutics, are located within kinase domains (3, 8-10). Therefore, we focused on single nucleotide variants (SNVs) present in the kinase domains of cancer-related RTKs to identify novel tumor-specific functional mutations from whole-exome sequencing (WES) data in Project HOPE.

Correspondence to: Kusuhara Masatoshi, MD, Ph.D., Drug Discovery and Development Division, Shizuoka Cancer Center Research Institute, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka 411-8777, Japan. Tel: +81 0559895222, Fax: +81 0559896085, e-mail: m.kusuhara@scchr.jp

Key Words: FLT3, kinase subdomain IX, molecular-targeted cancer therapeutics, NTRK1, receptor tyrosine kinase, whole-exome sequencing.

Moreover, additional filtering for the selection of SNVs with potential as functional mutations from SNVs detected within the RTK kinase domains is thought to be necessary for establishing efficient screening strategy of SNVs, which can then be subjected to functional evaluation. Thus, we focused on the fact that cancer progression results from somatic evolutionary events related to survival within normal cells (11). It was reported that the frequency of substitutions between amino acid pairs with different physicochemical properties, which can cause functional alterations, occurs with low frequency during the evolutionary process compared with that of substitutions between amino acid pairs with similar physicochemical properties (12, 13). Therefore, the evaluation from the molecular evolutionary perspective of substitution pattern between amino acid pairs in each SNV is thought to be reasonable as an additional filtering strategy. Miyata *et al*. (13) demonstrated that the amino acid pair distance value (d), indicating the degree of difference in physicochemical properties between amino acid pairs, which is calculated from the polarity and the volume of each amino acid, is significantly negatively correlated with the relative frequency of amino acid substitution during the evolutionary process. This observation indicates the possibility that this d value may not only represent physicochemical differences between amino acid pairs, but might also reflect the relative frequency of amino acid substitutions during the evolutionary process. Therefore, we implemented a filtering process for the selection of SNVs causing amino acid substitution between six different amino acid groups classified based on the similarity of d values reported by Miyata *et al*. (13). In fact, well-known oncogenic driver SNVs, such as L858R and T790M, in epidermal growth factor receptor (*EGFR*), G12 or G13C/R/D/V in Kirsten rat sarcoma viral oncogene homolog (*KRAS*), V600E in B-Raf proto-oncogene, serine/threonine kinase (*BRAF*), and E542K and E545K in phosphatidylinositol-4,5-*bis*phosphate 3-kinase catalytic subunit alpha (*PIK3CA*) (14), satisfy the criterion of this filtering process.

In this study, we describe the functional analysis of two novel tumor-specific SNVs located in the kinase domain of RTKs identified by using our strategy for the screening of SNVs further subjected to functional evaluation from the results of WES analysis performed through Project HOPE.

Materials and Methods

Patients and tissue samples. Surgically resected tumor specimens and corresponding peripheral blood samples used in this study were obtained from 220 consecutive patients who were admitted to the Shizuoka Cancer Center and enrolled in Project HOPE between January 2014 and August 2014. This study protocol was approved by the Institutional Review Board of the Shizuoka Cancer Center (approval number #25-33).

WES analysis. WES was performed on an Ion Proton system (Thermo Fisher Scientific, Waltham, MA, USA) using Ion Ampliseq Exome kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Data processing for the detection of tumor-specific mutations was performed with Ion Reporter software (Thermo Fisher Scientific) according to the manufacturer's instructions. Data visualization was performed with Integrated Genome Viewer (15).

Validation by Sanger sequencing. Sanger sequencing was performed to confirm the novel tumor-specific mutations detected in WES. The DNA sequencing template was amplified by polymerase chain reaction (PCR) with Hotstartaq DNA Polymerase (Qiagen, Venlo, the Netherlands) using 50 ng of genomic DNA and primers [5'-AATGATGGGGCTGGGGTAGG-3' and 5'-AAGGAACCTGAAG GGGCATG-3' for neurotrophic receptor tyrosine kinase 1 (*NTRK1*) V710A; 5'-GCACAAGCCTTTGTTCGAGA-3' and 5'-AGATCTG CCATGTGCCAGAC-3' for fms related tyrosine kinase 3 (*FLT3*) K868N], and subsequently purified using Illustra ExoProStar (GE Healthcare Life Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The cycle sequencing reaction and detection of nucleotide sequencing were performed at an outside independent laboratory (Takara Bio, Shiga, Japan). Primers for the amplification of each sequencing template were also used for cycle sequencing reactions. Output data were analyzed with CLC main workbench (CLC Bio, Aarhus, Denmark).

Construction of plasmids for the expression of mutant proteins. Plasmids for the expression of *NTRK1* V710A and *FLT3* K868N mutant proteins were constructed by site-directed mutagenesis with PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the manufacturer's instructions. *NTRK1* wild-type (NM_002529.3) and *FLT3* wild-type (NM_004119.2) expression plasmids were purchased from Genecopoeia (Rockville, MD, USA) and used as templates for site-directed mutagenesis. The sequences of the primers with the mutated nucleotide underlined used for site-directed mutagenesis are as follows: 5'-AGCGACGCGTGGAGCTTCGGCGTGGT-3' and 5'-GCTCCACGCGTCGCTCTCGGTGGTGA-3' for *NTRK1* V710A; 5'-CCATTAATAGTGATGTCTGGTCATAT-3' and 5'-CATCACTAT TAATGGTGTAGATGCCT-3' for *FLT3* K868N. The resultant mutant clones were subjected to Sanger sequencing to confirm the presence of the targeted mutations and to exclude clones with unintended mutations, which occurred during the mutagenesis reactions.

Cell culture. The human embryonic kidney cell line, HEK-293 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), was maintained in RPMI1640 (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific) in humidified air containing 5% carbon dioxide at 37˚C.

Immunoblot analysis. HEK-293 cells (4×10^5 cells/well) were seeded in 6-well plates (Corning Costar, Cambridge, MA, USA). After incubation for 24 h, cells were transfected with 2.5 μg/well of each expression plasmid using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. An empty plasmid, pReceiver-M02CT (Genecopoeia), was transfected as a negative control. Transfection with each plasmid was performed in four wells. After 32 h, the cultured cells were washed twice with ice-cold phosphate-buffered saline. Cells from the four

wells were collected in one tube and lysed using M-PER mammalian protein extraction reagent with an EDTA-free Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Thermo Scientific).

Equal amounts of protein (20 μg) in whole-cell lysate [as measured by using the BCA protein assay reagent (Thermo Scientific)] were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% skim milk or 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) for 1 hour at room temperature and then incubated overnight with primary antibodies in TBST containing 5% skim milk or 5% BSA at 4˚C. After three washes with TBST, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Promega, Madison, WI, USA) for 1 hour at room temperature and then subjected to three washes with TBST. Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). The images were captured using the ImageQuant LAS4000 system (GE Healthcare Life Sciences). The densitometric analysis was performed using ImageQuantTL software (GE Healthcare Life Sciences).

Antibodies against β-actin (sc-47778) and FLT3 (sc-20733) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against NTRK1 (2508), phospho-NTRK1 (4621), phospho-FLT3 (3464), v-akt murine thymoma viral oncogene homolog (AKT, 9272), phospho-AKT (4060), extracellular signal regulated kinases 1 and 2 (ERK1/2, 9102), and phospho-ERK1/2 (4370) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Luciferase assay. Serum Response Element (SRE) reporter assay (Qiagen) for the detection of serum response factor (SRF)-mediated transcriptional regulatory activity was performed according to the manufacturer's instructions. This luciferase reporter assay was designed to assess the activation status of ERK1/2 *via* the detection of the transcriptional regulatory activity of *SRF*, which is one of the genes regulated by the ERK pathway (16).

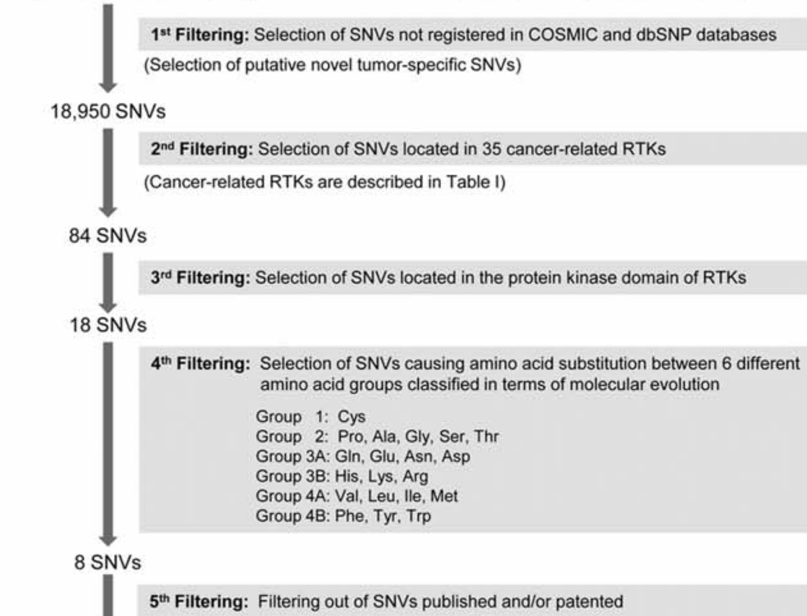
HEK-293 cells (1×10^5 cells/well) were seeded in 24-well plates (Corning Costar). After incubation for 24 hours, cells were transfected with 500 ng/well of each expression plasmid and 250 ng/well of either the SRE reporter mix composed of SRE reporter plasmid containing *Firefly* luciferase and *Renilla* luciferase plasmid or the negative control mix composed of empty reporter plasmid and *Renilla* luciferase plasmid, using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfection efficiency was normalized by the intensity of the co-transfected *Renilla* luciferase plasmid. After 32 h of incubation, cells were lysed in Reporter Lysis Buffer (Promega) and luciferase activity was assessed using the Dual-Glo luciferase assay system (Promega) with GLOMAX multi detection system (Promega).

Multiple amino acid sequence alignment. Multiple alignments were performed on CLC main workbench (CLC Bio) by using neighbor-joining (NJ) methods.

Statistical analysis. Welch's *t*-test was performed to assess the results of SRE reporter assay. A *p*-value lower than 0.05 indicated statistically significant differences.

Results

Selection of SNVs subjected to functional evaluation from novel tumor-specific SNVs detected in WES analysis. SNVs subjected to functional evaluation in this study were selected according to the procedure described in Figure 1A. The WES analysis of samples from 220 patients resulted in the detection of 27,049 non-synonymous tumor-specific SNVs. Among them, 18,950 SNVs were selected as putative novel tumor-specific SNVs by filtering out SNVs registered in the Catalogue of Somatic Mutations in Cancer (COSMIC) (17) or dbSNP (18) databases. Among the selected novel tumor-specific SNVs, 84 were located in 35 cancer-related RTKs defined based on the HUGO Gene Nomenclature Committee (19) and multiple cancer-related databases (3, 6, 14, 17, 20-24) (Table I). Eighteen SNVs within the protein kinase domain of RTKs were selected based on the Universal Protein Resource (Uniprot) database (25). As the subsequent filtering process, the evaluation from the molecular evolutionary perspective of substitution pattern between amino acid pairs in each SNV was performed. Accordingly, we selected eight SNVs causing amino acid substitution between six different amino acid groups classified by Miyata *et al*. (13). Finally, based on the surveillance of publication and patent information relevant to the eight SNVs, erb-b2 receptor tyrosine kinase 3 (*ERBB3*) Q809R was omitted from candidates subjected to further functional evaluation. Although this SNV was not registered in either the latest COSMIC (17) or dbSNP (18), it has been reported as a driver mutation capable of ligand-independent activation of downstream signaling cascades in gastric cancer (26, 27), indicating the adequacy of our strategy for the selection of mutations with potential as functional mutations from WES data. Protein kinase domains were further divided into 12 subdomains defined as regions containing the conserved functionally important amino acid residues (28). Among the remaining seven SNVs, after our 5-step filtering process, two, SNVs were located in subdomain III, one in VIA, three in IX, and one in X, respectively (Figure 1A). Thus, we focused on three SNVs, *NTRK1* V710A, neurotrophic receptor tyrosine kinase 2 (*NTRK2*) V752A, and *FLT3* K868N of subdomain IX, where most SNVs were detected in this study (Figure 1A). This subdomain IX is known to play an important role in the conformational stability of the catalytic loop of the active site (28). Because *NTRK1* V710 corresponds to *NTRK2* V752 and these two SNVs present the same amino acid substitution (Val to Ala) (Figure 1B), *NTRK1* V710A and *FLT3* K868N were functionally characterized in this study. These two SNVs were detected in patients with lung adenocarcinoma and gastric adenocarcinoma, respectively, with adequate mutant frequency in WES analysis (Figure 2A and B, left panel). Moreover, these SNVs were confirmed by conventional Sanger sequencing in tumor samples, but not in peripheral blood samples, indicating that these SNVs were reliable tumor-specific SNVs (Figure 2A


A
27,049 SNVs (Non-synonymous tumor-specific SNVs detected in 220 patients with cancer)
1st Filtering: Selection of SNVs not registered in COSMIC and dbSNP databases
(Selection of putative novel tumor-specific SNVs)
18,950 SNVs
2nd Filtering: Selection of SNVs located in 35 cancer-related RTKs
(Cancer-related RTKs are described in Table I)
84 SNVs
3rd Filtering: Selection of SNVs located in the protein kinase domain of RTKs
18 SNVs
4th Filtering: Selection of SNVs causing amino acid substitution between 6 different amino acid groups classified in terms of molecular evolution
Group 1: Cys
Group 2: Pro, Ala, Gly, Ser, Thr
Group 3A: Gln, Glu, Asn, Asp
Group 3B: His, Lys, Arg
Group 4A: Val, Leu, Ile, Met
Group 4B: Phe, Tyr, Trp
8 SNVs
5th Filtering: Filtering out of SNVs published and/or patented
7 SNVs
Candidate SNVs subjected to functional evaluation
Kinase subdomain
No. of SNVs
III 2
VIA 1
IX 3
X 1
NTRK1 V710A
NTRK2 V752A
FLT3 K868N

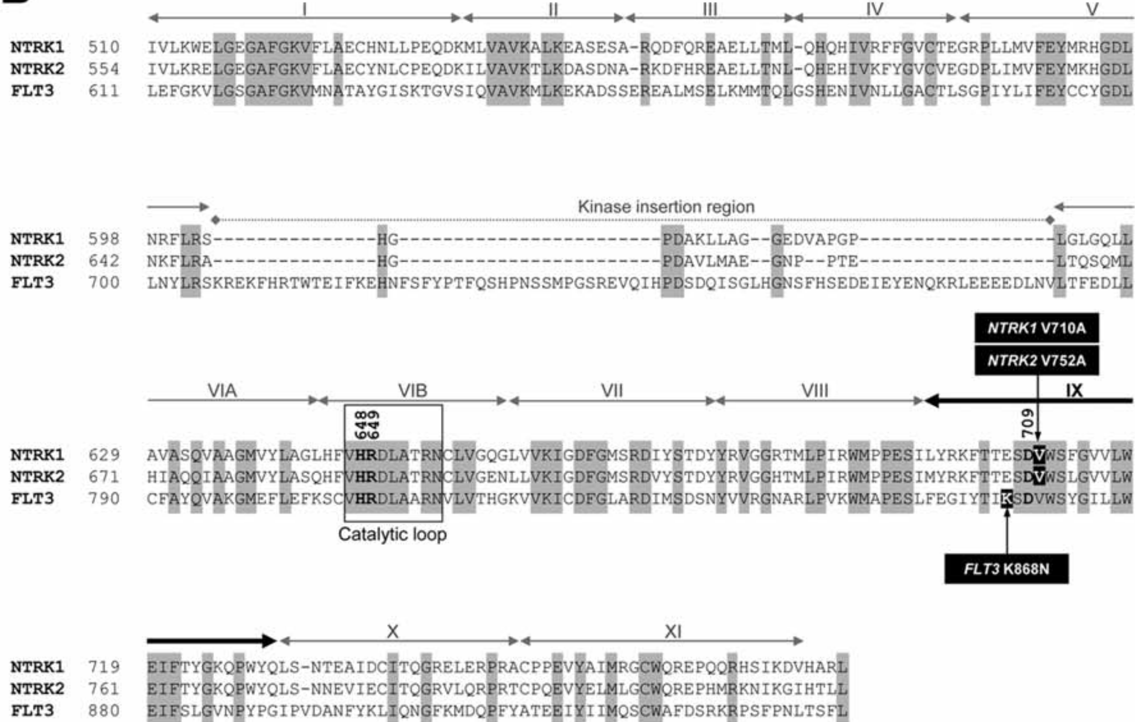

B
I
II
III
IV
V
NTRK1 510 IVLKWELGEGAFGKVFLAECHNLLPEQDKMLVAVKALKEASESA-RQDFQREAELLTML-QHQHIVRFFGVCTEGRPLLMVFEYMRHGDL
NTRK2 554 IVLKRELGEGAFGKVFLAECYNLCPEQDKILVAVKTLKDASDNA-RKDFHREAELLTNL-QHEHIVKFYGVCVEGDPLIMVFEYMKHGDL
FLT3 611 LEFGKVLGSGAFGKVMNATAYGISKTGVSIQVAVKMLKEKADSSEREALMSELKMMTQLGSHENIVNLLGACTLSGPIYLIFEYCCYGDL
Kinase insertion region
NTRK1 598 NRFLRS---------------HG------------------------PDAKLLAG--GEDVAPGP------------------LGLGQLL
NTRK2 642 NKFLRA---------------HG------------------------PDAVLMAE--GNP--PTE------------------LTQSQML
FLT3 700 LNYLRSKREKFHRTWTEIFKEHNFSFYPTFQSHPNSSMPGSREVQIHPDSDQISGLHGNSFHSEDEIEYENQKRLEEEEDLNVLTFEDLL
NTRK1 V710A
NTRK2 V752A
VIA
VIB
VII
VIII
IX
648
649
709
NTRK1 629 AVASQVAAGMVYLAGLHFVHRDLATRNCLVGQGLVVKIGDFGMSRDIYSTDYYRVGGRTMLPIRWMPPESILYRKFTTESDVWSFGVVLW
NTRK2 671 HIAQQIAAGMVYLASQHFVHRDLATRNCLVGENLLVKIGDFGMSRDVYSTDYYRVGGHTMLPIRWMPPESIMYRKFTTESDVWSLGVVLW
FLT3 790 CFAYQVAKGMEFLEFKSCVHRDLAARNVLVTHGKVVKICDFGLARDIMSDSNYVVRGNARLPVKWMAPESLFEGIYTIKSDVWSYGILLW
Catalytic loop
FLT3 K868N
X
XI
NTRK1 719 EIFTYGKQPWYQLS-NTEAIDCITQGRELERPRACPPEVYAIMRGCWQREPQQRHSIKDVHARL
NTRK2 761 EIFTYGKQPWYQLS-NNEVIECITQGRVLQRPRTCPQEVYELMLGCWQREPHMRKNIKGIHTLL
FLT3 880 EIFSLGVNPYPGIPVDANFYKLIQNGFKMDQPFYATEEIYIIMQSCWAFDSRKRPSFPNLTSFL

Table I. *Cancer-related receptor tyrosine kinase genes defined in this study based on multiple cancer-related databases.*

Gene symbol	Gene name	References
ALK	anaplastic lymphoma receptor tyrosine kinase	(3, 6, 14, 17, 20-24)
AXL	AXL receptor tyrosine kinase	(17, 22, 24)
CSF1R	colony-stimulating factor 1 receptor	(3, 17, 20-24)
DDR1	discoidin domain receptor tyrosine kinase 1	(17, 24)
DDR2	discoidin domain receptor tyrosine kinase 2	(14, 17, 21, 22, 24)
EPHA2	EPH receptor A2	(17, 24)
EPHA3	EPH receptor A3	(17, 21-24)
EPHA5	EPH receptor A5	(17, 21-24)
EPHB1	EPH receptor B1	(17, 22, 24)
EPHB2	EPH receptor B2	(17, 24)
EPHB6	EPH receptor B6	(23, 24)
EGFR	epidermal growth factor receptor	(3, 6, 14, 17, 20-24)
ERBB2	erb-b2 receptor tyrosine kinase 2	(3, 6, 14, 17, 20-24)
ERBB3	erb-b2 receptor tyrosine kinase 3	(17, 21-24)
ERBB4	erb-b2 receptor tyrosine kinase 4	(17, 20-24)
FGFR1	fibroblast growth factor receptor 1	(14, 17, 20-24)
FGFR2	fibroblast growth factor receptor 2	(3, 6, 14, 17, 20-24)
FGFR3	fibroblast growth factor receptor 3	(3, 6, 17, 20-24)
FGFR4	fibroblast growth factor receptor 4	(17, 21-24)
FLT1	fms-related tyrosine kinase 1	(17, 22-24)
FLT3	fms-related tyrosine kinase 3	(3, 6, 14, 17, 20-24)
FLT4	fms-related tyrosine kinase 4	(6, 17, 22-24)
IGF1R	insulin like growth factor 1 receptor	(17, 21-24)
INSR	insulin receptor	(17, 22)
KDR	kinase insert domain receptor	(17, 20-24)
KIT	kIT proto-oncogene receptor tyrosine kinase	(3, 6, 14, 17, 20-24)
LTK	leukocyte receptor tyrosine kinase	(17, 21)
MET	MET proto-oncogene, receptor tyrosine kinase	(3, 6, 14, 17, 20-24)
NTRK1	neurotrophic receptor tyrosine kinase 1	(6, 14, 17, 21-24)
NTRK2	neurotrophic receptor tyrosine kinase 2	(17, 21-24)
NTRK3	neurotrophic receptor tyrosine kinase 3	(17, 21-24)
PDGFRA	platelet-derived growth factor receptor alpha	(3, 6, 14, 17, 20-24)
PDGFRB	platelet-derived growth factor receptor beta	(17, 21-24)
RET	RET proto-oncogene	(3, 6, 14, 17, 20-24)
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase	(6, 14, 17, 21, 22, 24)

Gene symbol and gene name were described according to the HUGO Gene Nomenclature Committee (19).

←

Figure 1. *Strategy for the selection of single nucleotide variants (SNVs) subjected to further functional evaluation from 27,049 SNVs detected in whole-exome sequencing (WES) analysis of samples from 220 patients with cancer. This study aimed to identify novel functional tumor-specific SNVs in the kinase domain of receptor tyrosine kinases (RTKs) from the results of WES analysis of 220 patients with cancer. A: A 5-step filtering process was performed, resulting in the selection of seven novel tumor-specific SNVs as candidate SNVs for further functional evaluation. The amino acid classification used in the fourth filtering process was based on the approach of Miyata et al. (13). Three SNVs, neurotrophic receptor tyrosine kinase 1 (NTRK1) V710A, neurotrophic receptor tyrosine kinase 2 (NTRK2) V752A, and fms related tyrosine kinase 3 (FLT3) K868N, were located in kinase subdomain IX. B: Multiple amino acid alignment of the kinase domains of NTRK1, NTRK2, and FLT3 genes. Amino acid residues conserved between the three genes are shaded. Twelve protein kinase subdomains predicted according to Hanks et al. (28) are indicated above the alignments. The catalytic loops, predicted according to previous reports (28, 39, 40, 42) are boxed. Amino acid residues of kinase subdomain IX of NTRK1, NTRK2, and FLT3 are from amino acids 700 to 730, 742 to 772, and 861 to 891, respectively. NTRK1 V710A and NTRK2 V752A are corresponding amino acid substitutions. Based on the alignment between NTRK1, human insulin receptor, and mouse Fer protein kinase (data not shown), it was predicted that D709 in the kinase subdomain IX of NTRK1 forms hydrogen bonds with H648 and R649 residues within the kinase subdomain VIB.*

and B, right panel). NTRK1 is a receptor for nerve growth factor and a member of the NTRK family, which also includes NTRK2 and NTRK3. NTRK1 plays an important role in the development of sympathetic neurons (29). Moreover, because chromosomal rearrangements causing oncogenic fusion genes were reported in colorectal cancer (30), papillary thyroid cancer (31), and lung adenocarcinoma (32), NTRK1 was widely recognized as a promising therapeutic target. *FLT3* is best known as one of the driver genes in acute myeloid leukemia (AML) (33). Clinical trials of anticancer agents targeting FLT3 are in progress (34). An internal tandem duplication and mutations located in D835 are known oncogenic drivers in AML (35).

NTRK1 V710A and FLT3 K868N SNVs reduce autophosphorylation of each RTK. To evaluate the effect of *NTRK1* V710A and *FLT3* K868N on autophosphorylation of each RTK and the activity of downstream signaling pathways, wild-type and mutant proteins for each gene were transiently expressed in HEK-293 cells and immunoblot analysis was performed (Figure 3). HEK-293 cells have been used as a host cell system to evaluate the effect of mutations in RTKs on autophosphorylation and downstream signaling pathways (36, 37). In our preliminary analysis, we confirmed that overexpression of *FLT3* D835H/Y, which are known driver mutations in AML (35, 38), resulted in an increase in ERK1/2 phosphorylation (data not shown), indicating that this HEK-293-based bioassay was acceptable.

Cells expressing wild-type NTRK1 and FLT3 showed robust phosphorylation of each RTK (Figure 3A). Meanwhile, a reduction in the expression of phospho-NTRK1 and phospho-FLT3 was observed in cells expressing *NTRK1* V710A and *FLT3* K868N mutant proteins, respectively (Figure 3A and B). These results indicate that both SNVs present in kinase subdomain IX can cause a significant reduction of RTK catalytic activity, as determined by the autophosphorylation level.

NTRK1 V710A and FLT3 K868N cause a reduction in ERK1/2 activity. To investigate whether the reduction in the autophosphorylation of RTKs caused by *NTRK1* V710A and *FLT3* K868N affected the activity of the downstream ERK and phosphoinositol 3-kinase (PI3K)/AKT pathways, the effect of both SNVs on the phosphorylation status of ERK1/2 and AKT was evaluated by immunoblot analysis (Figure 3A). ERK1/2 phosphorylation was significantly reduced by the expression of both mutant proteins (Figure 3A and 3C), while no effect was observed on AKT phosphorylation (Figure 3A).

We further examined whether the reduction of ERK phosphorylation caused by both SNVs resulted in a decrease of ERK functional activity. It has been reported that ERK phosphorylation induces the formation of a transcription factor complex composed of ELK1, ETS transcription factor (ELK1) and SRF, and to the subsequent binding of this complex to SRE (16). Therefore, a luciferase assay to assess the SRF-mediated transcriptional regulatory activity as a surrogate marker reflecting ERK activity was performed by using the reporter, including SRE (Figure 4). The SRF-mediated transcriptional regulatory activity in cells expressing *NTRK1* V710A and *FLT3* K868N was significantly repressed compared to that in cells expressing the respective wild-type protein. In particular, *FLT3* K868N induced a significant reduction of SRF-mediated transcriptional regulatory activity when compared to that induced by *NTRK1* V710A (Figure 4). These results were consistent with our immunoblot analysis of phospho-ERK1/2 (Figure 3A and C). These results indicate that *NTRK1* V710A and *FLT3* K868N are functional mutations which cause a reduction of the ERK pathway activity.

Discussion

In this study, we demonstrated that two novel tumor-specific SNVs in kinase subdomain IX of RTKs, *NTRK1* V710A and *FLT3* K868N, caused a reduction of ERK pathway activity by inhibiting the kinase activity of NTRK1 and FLT3, respectively. Based on the surveillance of cancer-specific non-synonymous mutations in the kinase subdomain IX of NTRK1 and FLT3 with the latest COSMIC database v76 (17), 10 types of mutation were observed in each gene, respectively. However, there was no report regarding the functional effect of these registered mutations. Therefore, to our knowledge, this is the first report to demonstrate the presence of functional mutations in kinase subdomain IX of NTRK1 and FLT3.

Insights from a molecular structural perspective further the understanding of the molecular mechanisms underlying the functional conversion caused by *NTRK1* V710A and *FLT3* K868N. Regarding the kinase subdomain IX, the association between the mouse Fer protein kinase (*Fer*) D743R mutation, present on a conserved amino acid residue within kinase subdomain IX, and its kinase activity has been reported (39). Based on the results of the crystal structure analysis of the human insulin receptor, this Asp residue in subdomain IX of Fer has been predicted to form hydrogen bonds with H683 and R684 residues within the kinase catalytic loop, which is conserved among kinases (39, 40). The structural modeling of *Fer* D743R mutant protein predicted that this mutation caused the dramatic reorientation of H683 and R684, which are key amino acid residues within the catalytic loop, resulting in the abrogation of Fer kinase activity (39). These key amino acid residues, D743, H683, and R684, of Fer protein correspond to amino acids D709, H648, and R649 of NTRK1, respectively (Figure 1B). *NTRK1* V710A is in the right-adjacent position of an important Asp residue in kinase subdomain IX (D709), indicating the possibility that the reduction of autophosphorylation of NTRK1 observed in cells expressing *NTRK1* V710A mutant protein (Figure 3A and B) might be

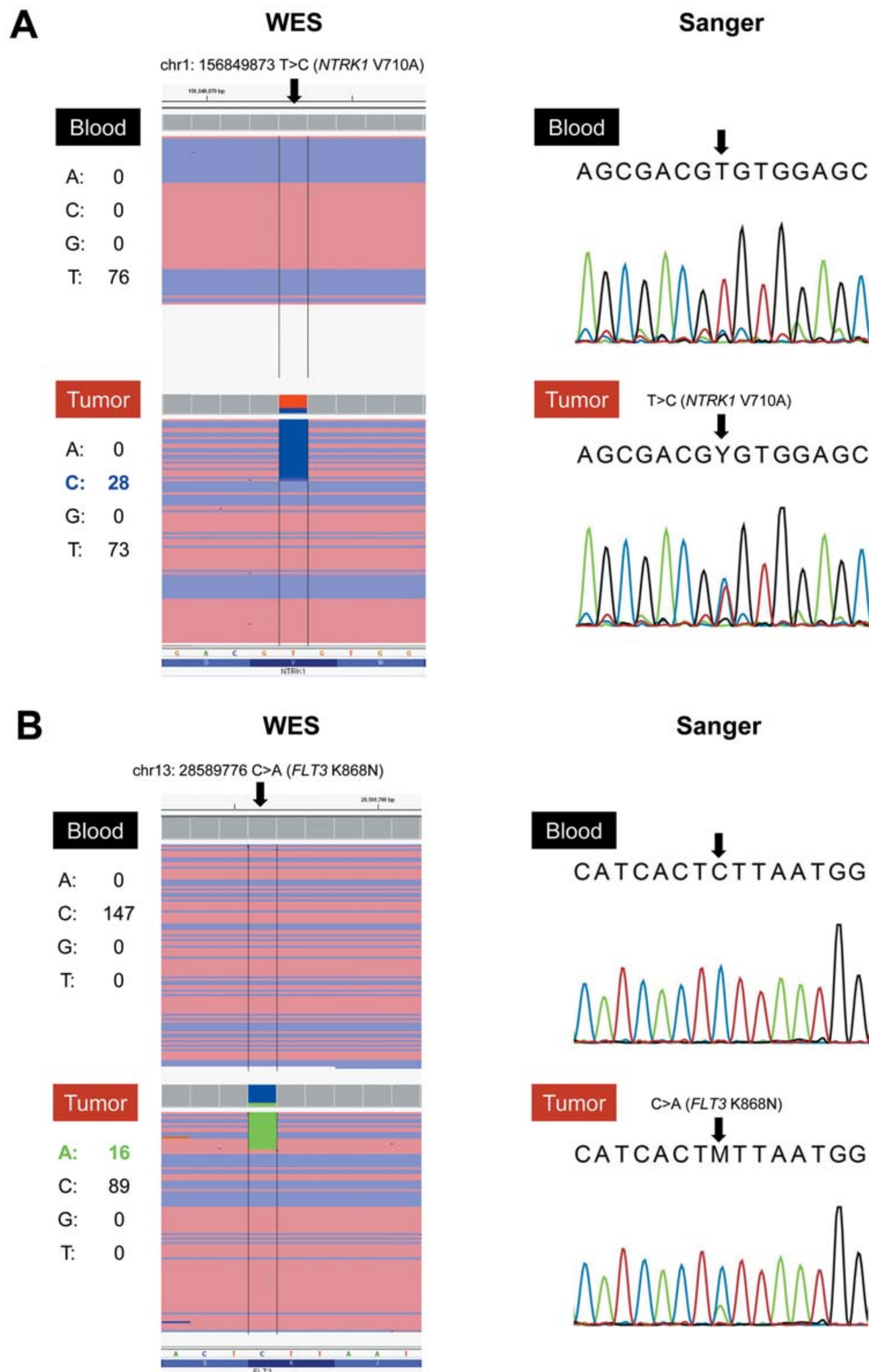
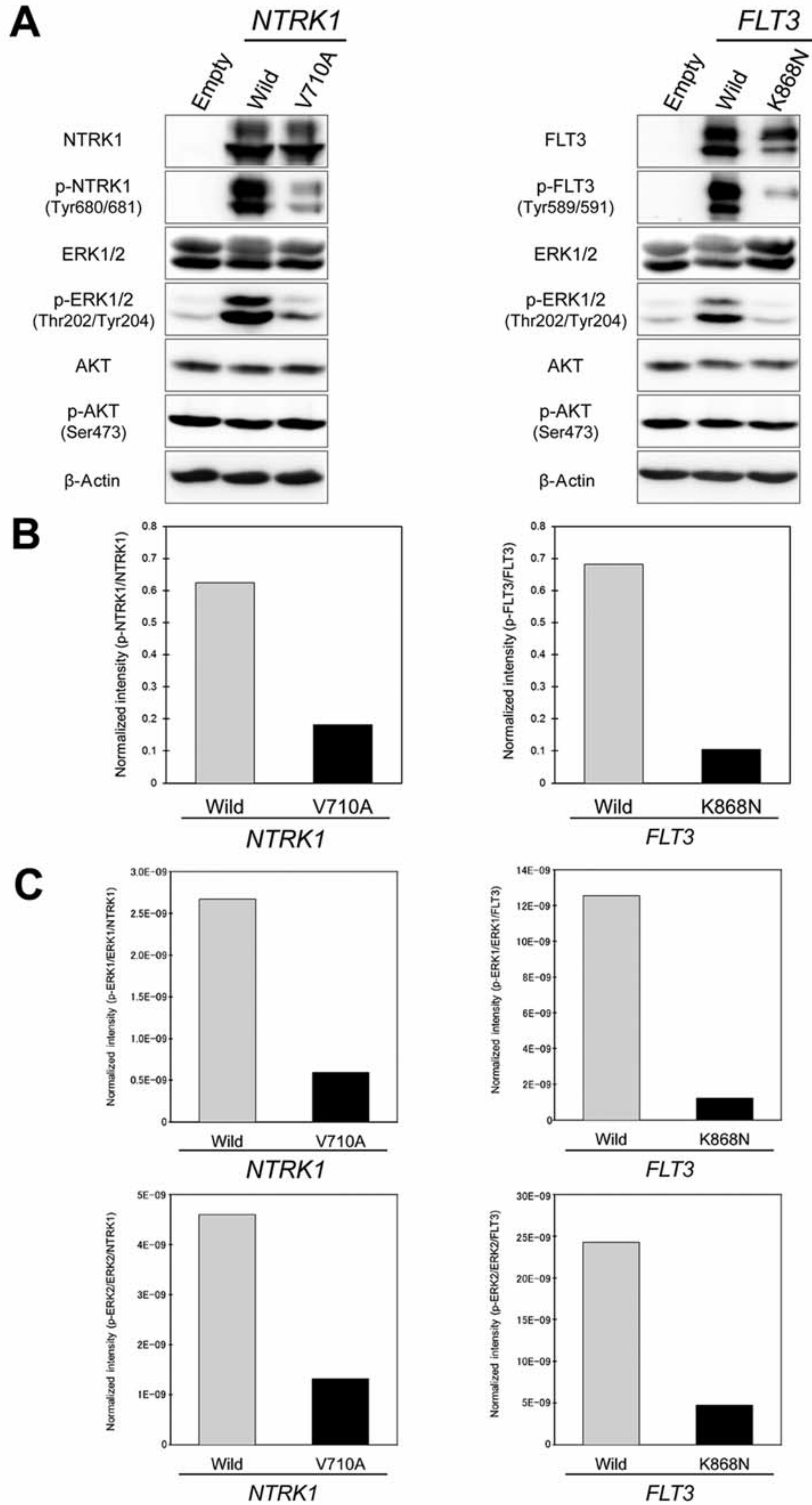


Figure 2. *Identification of neurotrophic receptor tyrosine kinase 1 (NTRK1) V710A (A) and fms-related tyrosine kinase 3 (FLT3) K868N (B) by whole-exome sequencing (WES) (left panel) and validation by Sanger sequencing (right panel). The results of WES analysis were visualized with Integrated Genome Viewer. The mutation frequencies in WES analysis for tumor samples harboring the NTRK1 V710A and FLT3 K868N mutation were approximately 28% and 15%, respectively. Both single nucleotide variants (SNVs) were confirmed by Sanger sequencing. Down-pointing arrows indicate each SNV position.*


A
NTRK1
Empty
Wild
V710A
NTRK1
p-NTRK1 (Tyr680/681)
ERK1/2
p-ERK1/2 (Thr202/Tyr204)
AKT
p-AKT (Ser473)
β-Actin
FLT3
Empty
Wild
K868N
FLT3
p-FLT3 (Tyr589/591)
ERK1/2
p-ERK1/2 (Thr202/Tyr204)
AKT
p-AKT (Ser473)
β-Actin
B
Normalized intensity (p-NTRK1/NTRK1)
Wild
V710A
NTRK1
Normalized intensity (p-FLT3/FLT3)
Wild
K868N
FLT3
C
Normalized intensity (p-ERK1/ERK1/NTRK1)
Wild
V710A
NTRK1
Normalized intensity (p-ERK1/ERK1/FLT3)
Wild
K868N
FLT3
Normalized intensity (p-ERK2/ERK2/NTRK1)
Wild
V710A
NTRK1
Normalized intensity (p-ERK2/ERK2/FLT3)
Wild
K868N
FLT3

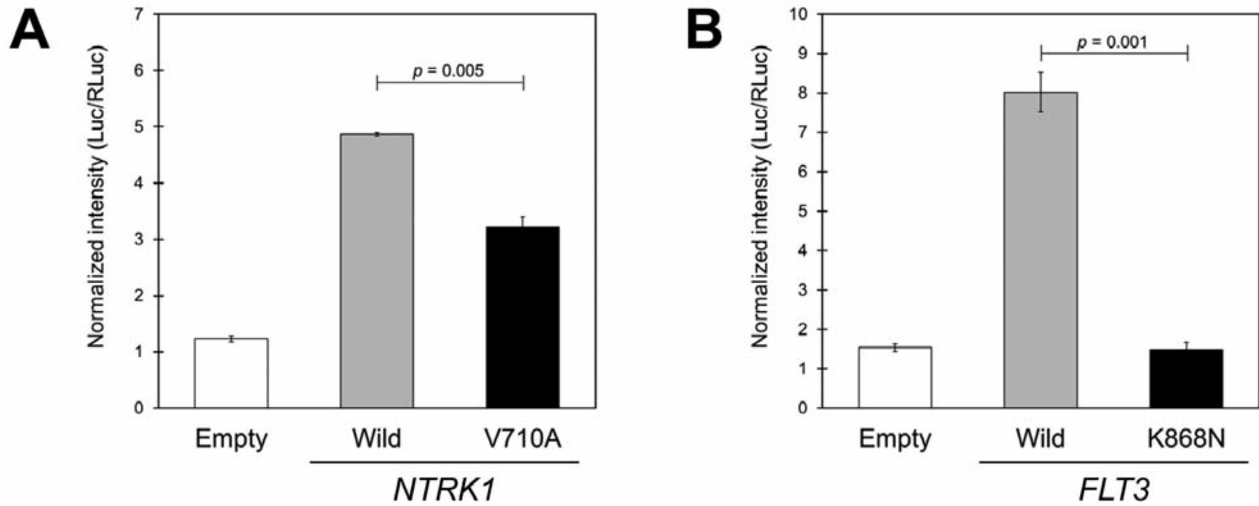


Figure 4. *Effects of neurotrophic receptor tyrosine kinase 1 (NTRK1) V710A and fms related tyrosine kinase 3 (FLT3) K868N mutations on the functional activity of extracellular signal regulated kinases 1 and 2 (ERK1/2) by evaluating serum response factor (SRF)-mediated transcriptional regulatory activity. Results of Luciferase assay with Serum Response Element (SRE) luciferase reporter (Luc) plasmid. Cells transfected with wild-type or V710A mutant NTRK1 (A) or with wild-type or K868N mutant FLT3 (B). An empty plasmid, pReceiver-M02CT, was transfected as a negative control. The values were normalized to the Renilla luciferase (RLuc) activity of a co-transfected reference plasmid. Error bars indicate standard deviation of the mean of three separate wells. p-Values were calculated with Welch's t-test. SRF-mediated transcriptional regulatory activity was reduced by the expression of NTRK1 V710A and FLT3 K868N.*

caused by the effects of V710A on the predicted hydrogen bonds between amino acid residues of the catalytic loop (H648 and R649) and D709 (Figure 1B). Moreover, among 35 cancer-related kinases, the conservation of amino acid residues corresponding to Ser-Asp-Val-Trp-Ser residues between 708 and 712 of NTRK1 is over 90% (Figure 1B and Figure 5). These observations imply that these consecutive amino acid residues may coordinately contribute to the stabilization of hydrogen bonds between the catalytic loop and kinase subdomain IX. Thus, kinase inhibitors targeting these amino acid residues might effectively inhibit the kinase activity.

Although the *FLT3* K868N mutation is observed on an amino acid residue that shows more diversity when compared to the conserved amino acid on which the *NTRK1* V710A is detected (Figure 5), the *FLT3* K868N mutation resulted in a potent reduction of FLT3 autophosphorylation and ERK activity when compared to the effects of *NTRK1* V710A (Figures 3 and 4), implying that this FLT3 Lys residue might be specifically important for FLT3 kinase activity. Moreover, no cancer-related RTK presented the same amino acid residues in positions corresponding to FLT3 I867 and K868 residues (Figure 5). Thus, these Ile-Lys residues may be considered as candidate target positions for the molecular design of FLT3-specific inhibitor.

Mutations located in kinase subdomain IX of RTKs have rarely been investigated as therapeutic targets compared to those in other kinase subdomains. In this study, we identified two novel tumor-specific functional mutations in kinase subdomain IX: *NTRK1* V710A and *FLT3* K868N. These SNVs were inactivating mutations that decreased the autophosphorylation of the respective RTK. A previous study indicated that inactivating mutations do not serve as molecular targets for RTK kinase inhibitors (36). However, we emphasize that the accumulation of information on inactivating mutations

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Figure 3. *Effects of neurotrophic receptor tyrosine kinase 1 (NTRK1) V710A and fms related tyrosine kinase 3 (FLT3) K868N on autophosphorylation of each receptor tyrosine kinase (RTK) and downstream signaling pathways. A: Cells transfected with empty plasmid, wild-type plasmid, or mutant plasmid were lysed and the indicated proteins were detected using immunoblot analysis. B: Densitometric analysis of phospho-NTRK1 expression in cells transfected with wild-type or V710A mutant NTRK1 (left panel) and of phospho-FLT3 expression in cells transfected with wild-type or K868N mutant FLT3 (right panel). Expression of NTRK1 V710A and FLT3 K868N reduced the autophosphorylation of NTRK1 and FLT3, respectively. C: Densitometric analysis of phospho-extracellular signal-regulated kinase 1 (ERK1) (upper panel) and phospho-extracellular signal-regulated kinase 2 (ERK2) (lower panel) expression in cells transfected with wild-type or V710A mutant NTRK1 (left panel) and in cells transfected with wild-type or K868N mutant FLT3 (right panel). Expression of NTRK1 V710A and FLT3 K868N resulted in a reduction of ERK1/2 phosphorylation. AKT: v-akt murine thymoma viral oncogene homolog.*

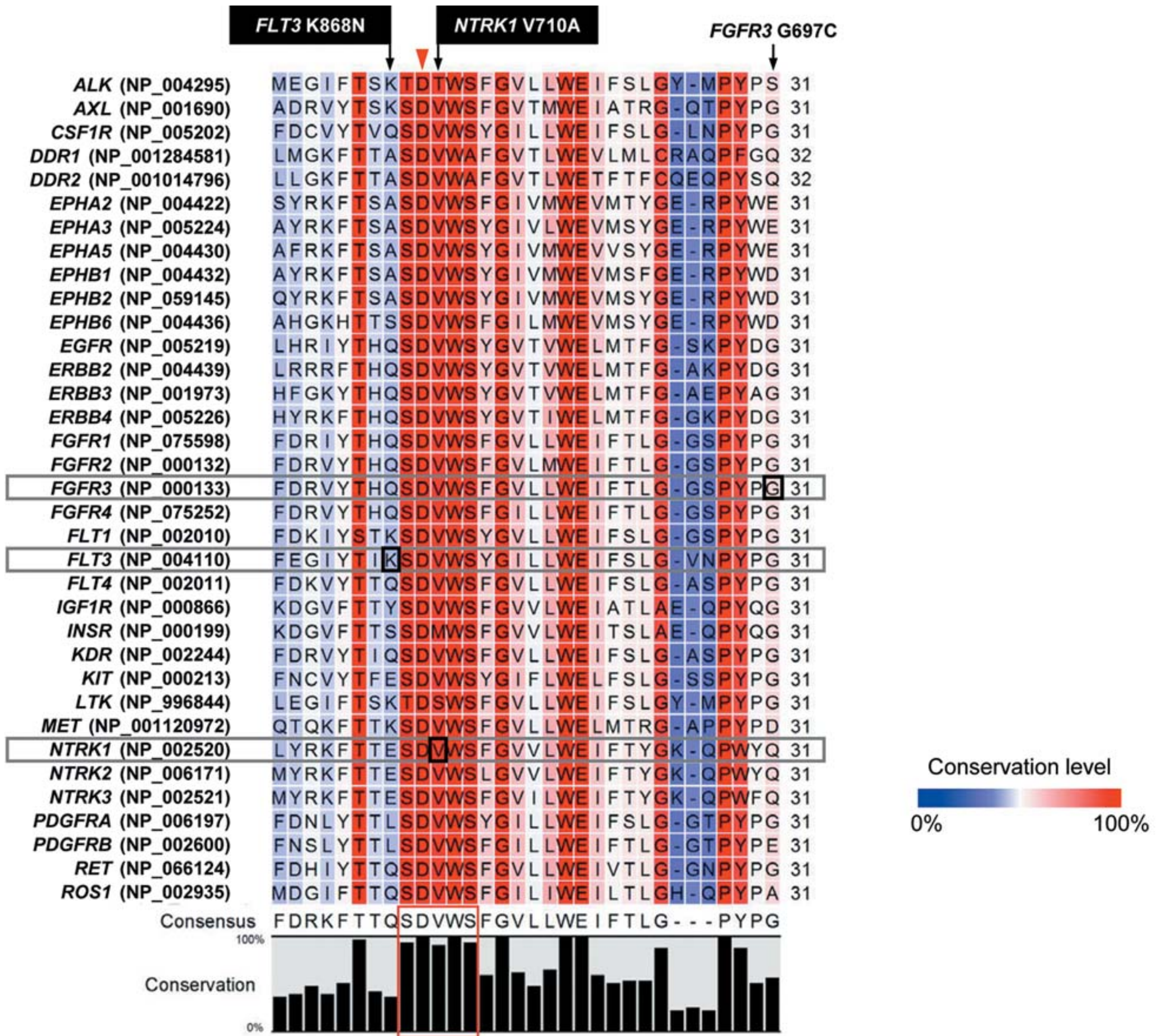

Figure 5. Comparison of amino acid residues within kinase subdomain IX encoded by 35 cancer-related receptor tyrosine kinases (RTKs) defined in this study. Multiple alignment of amino acid residues within kinase subdomain IX encoded by 35 cancer-related RTKs (Table I). The conservation level for each residue is indicated below the alignment. Arrowhead indicates the neurotrophic receptor tyrosine kinase 1 (NTRK1) D709 residue corresponding to mouse Fer protein kinase (Fer) D743, which has been predicted to form hydrogen bonds with amino acid residues within the kinase catalytic loop.

can contribute to the molecular design of low-molecular-weight kinase inhibitors as described above. Among tumor-specific SNVs located in kinase subdomain IX of 35 cancer-related RTKs (Table I, Figure 5), there is also a mutation reported as an oncogenic driver. It has been reported that fibroblast growth factor 3 isoform b (*FGFR3B*) G697C, which is observed in 62% of oral squamous cell carcinomas, causes constitutive activation of ligand-independent FGFR3B signaling (Figure 5) (41). Therefore, in order to facilitate the development of kinase inhibitors targeting kinase subdomain IX, further evaluation of the effects of each amino acid residue of kinase subdomain IX on RTK catalytic activity and the structural interaction with other kinase subdomains, is necessary.

In order to select mutations with potential as oncogenic drivers from WES data, we need to further improve the strategy for the selection of mutations subjected to functional evaluations by combination of additional omics data, such as transcriptomics and metabolomics, in addition to the strategy used in this study. Moreover, one of the limitations of this study is that because we could not generate suitable cell lines

stably expressing *NTRK1* V710A and FLT3 K868N mutant proteins, the evaluation of the effects of these SNVs on cell growth could not be demonstrated. Selection or efficient generation of cell lines appropriate for such evaluation is a common problem in the functional evaluation of novel tumor-specific genetic alterations. Therefore, in order to facilitate the identification of additional therapeutic targets, we plan to implement a system for generating mutated cells by genomic editing and a phenotype-based screening system using metabolomics data.

Acknowledgements

The Authors thank the patients who participated in this study and their families. The Authors also thank Akane Naruoka, Junko Saito, Rina Umehara, Tamiko Usui, and Tomoe Tanabe for technical assistance, as well as the staff of the Shizuoka Cancer Center Hospital for clinical support and sample preparation.

Conflicts of Interest

The Authors have no conflict of interest to declare in regard to this study.

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Received March 17, 2016
Revised April 28, 2016
Accepted May 8, 2016