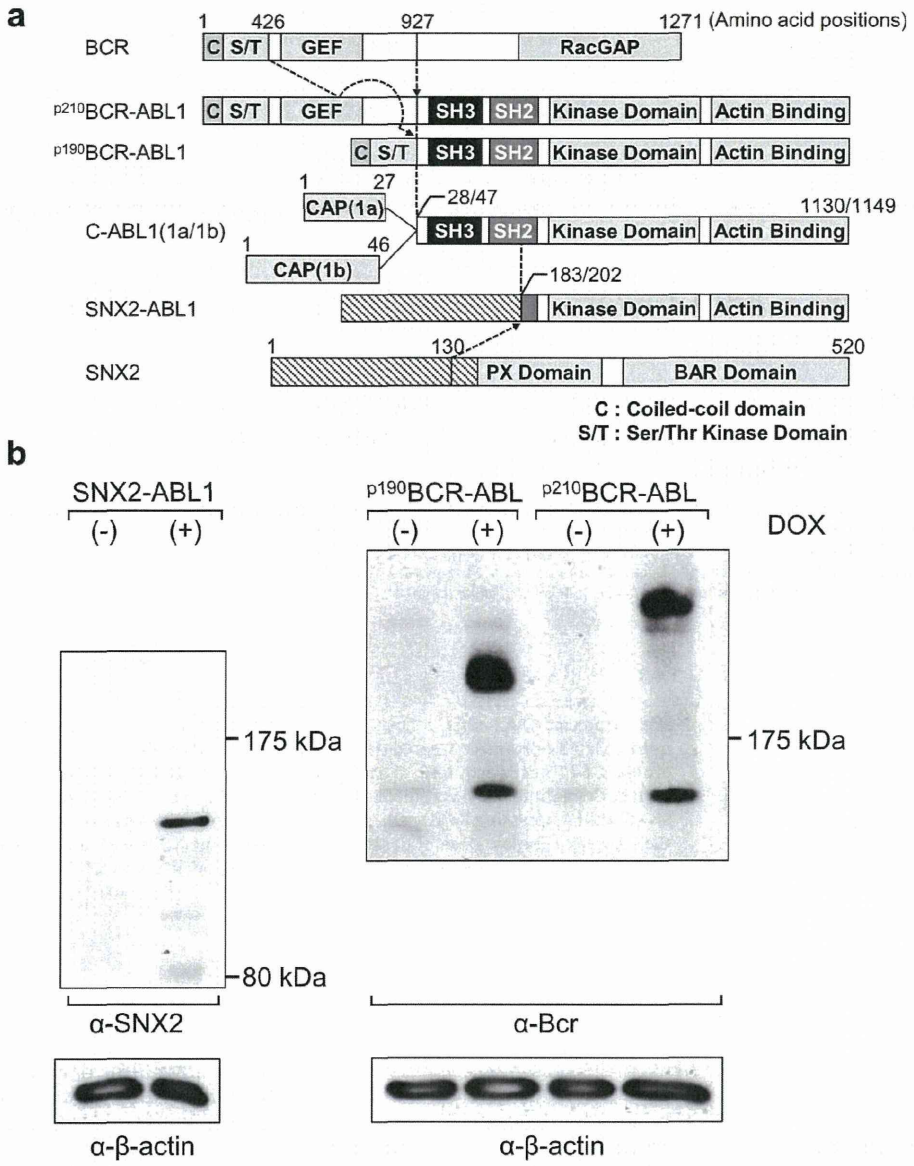

Fig. 1. Structure and expression of SNX2-ABL1 kinase. (a) Structures of SNX2-ABL1, ^{p190}BCR-ABL1 and ^{p210}BCR-ABL1 kinases are schematically presented. Amino acid positions of recombination sites are also indicated. (b) Doxycycline-induced protein expressions of SNX2-ABL1 and $^{p190/p210}$BCR-ABL1 in Ba/F3 cells were detected by immunoblotting using the indicated antibodies. Molecular mass references (kDa) are indicated. Simultaneously, β-actin was blotted as a loading control.

structure of imatinib with modifications added to improve binding and potency against BCR-ABL1 kinase and exhibits a stronger efficacy than imatinib [15,21]. Ponatinib (IC50: 0.37 nM) is a third-generation TKI optimized using structure-based drug design to bind to the inactive form of BCR-ABL1 [22] and rebastinib (IC50: 0.8 nM) acts via a non-ATP competitive mechanism and prevents the activation of ABL1-kinase by blocking an essential conformational change in the switch control pockets [23,24]. These TKIs have been approved for various settings of Ph+ leukemias [25].

On the other hand, there are a number of reports on *ABL1*-related chimeric genes that have various fusion partners other than *BCR* [4,5,26–33] such as *ETV6*, *NUP214*, *EML1*, and *RCSD1* and the resulting ABL1-related chimeric proteins should also show sensitivity to TKIs. *SNX2-ABL1* is a novel *ABL1*-related chimeric transcript first reported by Ernst et al. in which *SNX2* exon 3 was fused in frame to *ABL1* exon 4 [26]. The *SNX2* gene encodes sorting nexin (SNX) 2 that belongs to the SNX family of proteins, a diverse group of cellular trafficking proteins unified by the presence of a phospholipid-binding motif and involved in various aspects of endocytosis and protein trafficking [34,35]. SNX2 is an oligomeric protein containing a variety of domains for protein–protein and protein–lipid interactions, such as coiled-coil domains, and reported to be interact with a number of growth factor receptors including epidermal growth factor receptor and c-Met [36]. It is also logical to speculate that *SNX2-ABL1* is related to the pathogenesis of ALL, and that TKIs will be effective for ALL cells expressing this chimeric transcript as a therapeutic agent, but detailed data are not available.

We recently encountered a pediatric ALL patient with *SNX2-ABL1* chimeric transcripts who exhibited resistance to conventional chemotherapy and poorly responded to dasatinib but partially responded to imatinib [37], suggesting that treatment using TKIs requires further investigation to optimize the genotype-based treatment stratification of patients with *SNX2-ABL1* fusion. Therefore, we intend to investigate the functional characteristics of

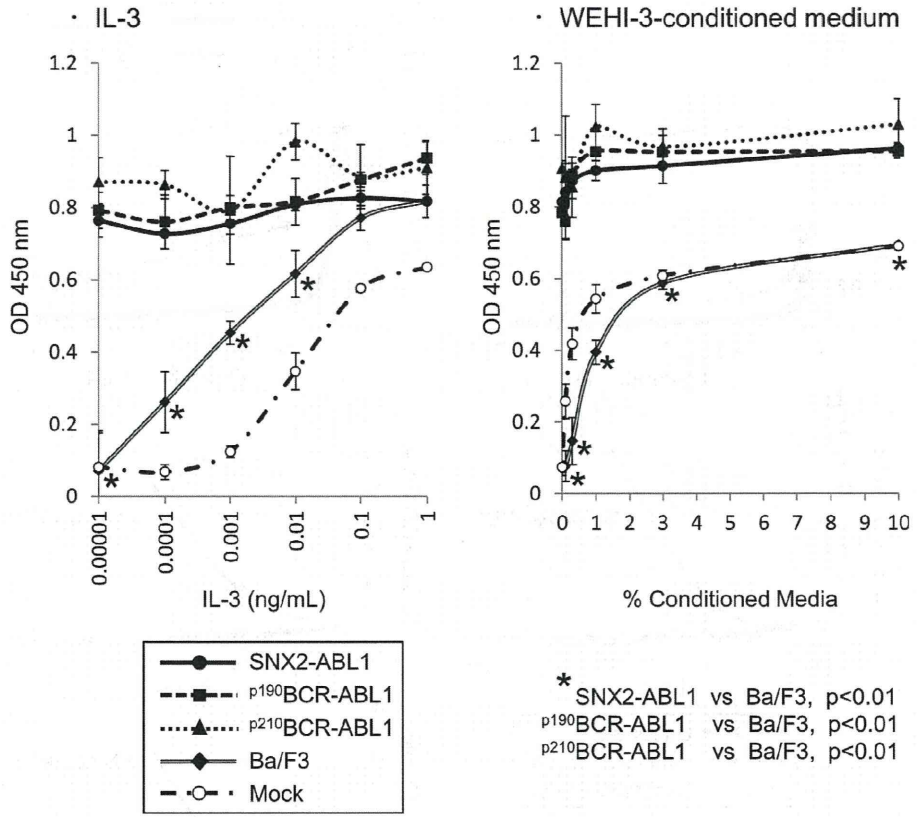

Fig. 2. Proliferation of Ba/F3 cells expressing SNX2-ABL1 and BCR-ABL1. Ba/F3 cells transfected with *SNX2-ABL1* and *BCR-ABL1* were cultured in the presence and absence of different concentrations of IL-3 or WEHI-3-conditioned medium, and were examined with the WST assay as described in Section 2. OD values are shown. SNX2-ABL1, SNX2-ABL1-expressing Ba/F3 cells; $^{p190/p210}$BCR-ABL1, $^{p190/p210}$BCR-ABL1-expressing Ba/F3 cells.

SNX2-ABL1 protein and its role in the development of ALL in comparison with that of BCR-ABL1 proteins. In this study, we report that SNX2-ABL1 protein is indeed functionally related to the pathogenesis of ALL and it shows poor sensitivity toward TKIs compared to that of BCR-ABL1 protein.

2. Materials and methods

2.1. Cells and cell culture

Ba/F3 cells (RIKEN Bioresource Center, Tsukuba, Ibaraki, Japan), K562 cells (Japanese Cancer Research Resource Bank, JCRB, Tokyo, Japan), and NALM-20 cells (Research Center Cell Biology Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) were maintained in standard RPMI 1640 medium (Sigma–Aldrich Corp., St. Louis, MO, USA) supplemented with 5% (vol/vol) fetal bovine serum (FBS; MP Biomedicals, Illkirch, France). In the case of Ba/F3 cells, 10% (vol/vol) conditioned medium from the WEHI-3 cell line (RIKEN) or 100 ng/mL purified mouse recombinant IL-3 (PeproTech EC Ltd., London, UK) were added. WEHI-3 cells were maintained in standard D-MEM (Sigma–Aldrich) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. The transformed embryonal kidney cell line 293FT (Invitrogen, Carlsbad, CA, USA) was also used and cultured in D-MEM with 10% (vol/vol) FBS.

2.2. Plasmid construction

The cloning of cDNAs was performed using the following primers: BCR-ABL1 (5′) forward (Fwd) 5′-CGCGCCATGG TGGACCCGGTGGGCTT-3′, BCR-ABL1 (5′) reverse (Rev) 5′-GATACTCAGCGGCATTGCGGGACACAGGCC-3′, SNX2-ABL1 (5′) Fwd 5′-ATGGCGGCCGAGAGGGAACCTCCTCCGCTG-3′, SNX2-ABL1 (5′) Rev 5′-TGTTGTAGGCCAGGCTCTCGGGTGCAGTCC-3′, ABL1 (3′) Fwd 5′-ACGCCAGTCAACAGTCTGGAGAAACACTCC-3′, ABL1 (3′) Rev 5′-CTACCTCTGCACTATGTCACTGATTTCCTT-3′.

The *major* (e14-a2) *^{p210}BCR-ABL1* (5′) and common *ABL1* (3′) were separately amplified from cDNA prepared from K562 cells by PCR using KOD plus ver.2 (Toyobo Co., Ltd., Osaka, Japan) and cloned into pGEM-T (Promega, Madison, WI, USA). The *minor* (e1-a2) *^{p190}BCR-ABL1* (5′) and *SNX2-ABL1* (5′) were similarly amplified from cDNAs prepared from NALM-20 cells and clinical specimens, respectively. pGEM-T-ABL1 (3′) is common among the three chimera genes. The resulting pGEM-T-major ^{p210}BCR-ABL1 (5′) or pGEM-T-minor ^{p190}BCR-ABL1 (5′) and pGEM-T-ABL1 (3′) were combined at the *KpnI* site to construct pGEM-T-major ^{p210}/minor ^{p190}BCR-ABL1. pGEM-T-SNX2-ABL1 (5′) and pGEM-T-ABL1 (3′) were combined at the *SacI* site to construct pGEM-T-SNX2-ABL1. Each plasmid was digested with *SphI* (blunted) and *NotI* and the insert was subcloned into the *BamHI* (blunted) and *NotI* sites of pRetroX Tight (Clontech Laboratories, Inc., Madison, WI, USA).

2.3. Transfection and induction of BCR-ABL1 and SNX2-ABL1

Tetracycline (Tet)-dependent chimera gene-inducible cell lines were generated by employing retroviral transfection using the Retro-X™ Tet-On® Advanced Inducible Expression System (Clontech), as described previously [38]. Tet-on Advanced-introduced Ba/F3 cells were further infected with retrovirus of pRetroX Tight major ^{p210}/minor ^{p190} BCR-ABL1, and SNX2-ABL1. The

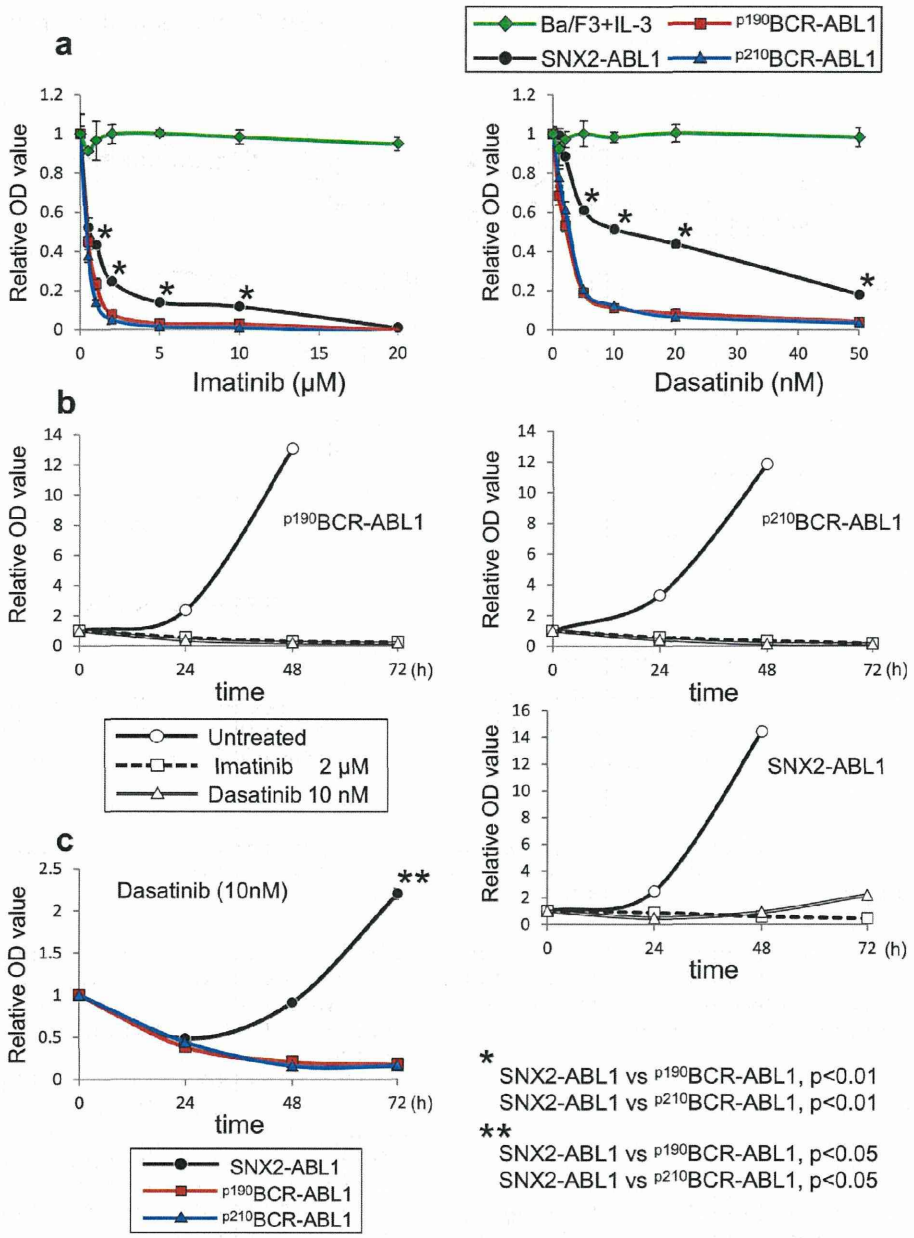

Fig. 3. Effect of imatinib and dasatinib on the proliferation of Ba/F3 cells expressing SNX2-ABL1 and BCR-ABL1. (a) Cell proliferation of Ba/F3 transfectants in the presence and absence of different concentrations of imatinib (left panel) and dasatinib (right panel) as indicated was examined by WST assay as in Fig. 2. Data are expressed as the ratio of the OD value against that obtained from untreated cells and the means ± SEMs of triplicates are indicated. (b) Ba/F3 transfectants were treated with 2 μM and 10 nM of imatinib and dasatinib, respectively, for the indicated time periods and examined as in (a). Data are expressed as the ratio of the OD value against that obtained from the initial cell number and the means ± SEMs of triplicates are indicated as in (a). (c) Data of Ba/F3 transfectants treated with 10 nM of dasatinib presented in (b) are superimposed. SNX2-ABL1, SNX2-ABL1-expressing Ba/F3 cells; $^{p190/p210}$BCR-ABL1, $^{p190/p210}$BCR-ABL1-expressing Ba/F3 cells.

transfectants were enriched by drug selection and proteins were induced by treatment with 1 μg/mL doxycycline (DOX).

2.4. Cell proliferation

Ba/F3 cells and chimeric gene-transfectants were washed twice with RPMI-1640 medium and dispensed in triplicate into 96-well plates (2.5×10^4 cells/0.1 ml per well) in the presence or absence of the indicated concentrations of WEHI-3 cell-conditioned medium, IL-3, and various TKIs, and were cultured for 24–72 h at 37 °C. Cell proliferation was analyzed using water-soluble tetrazolium salt (WST) assays (Cell Counting Kit-8, Dojindo, Kumamoto, Japan), as described previously [39]. Each experiment was performed in triplicate and the mean ± SEM were indicated. Experiments were repeated three times independently and representative data were presented.

2.5. Flow cytometric analysis

The frequency of apoptosis was quantified using MEBCYTO® Apoptosis Kit (Medical and Biological Laboratories, Nagoya, Japan), and then analyzed according to the manufacturer's protocol.

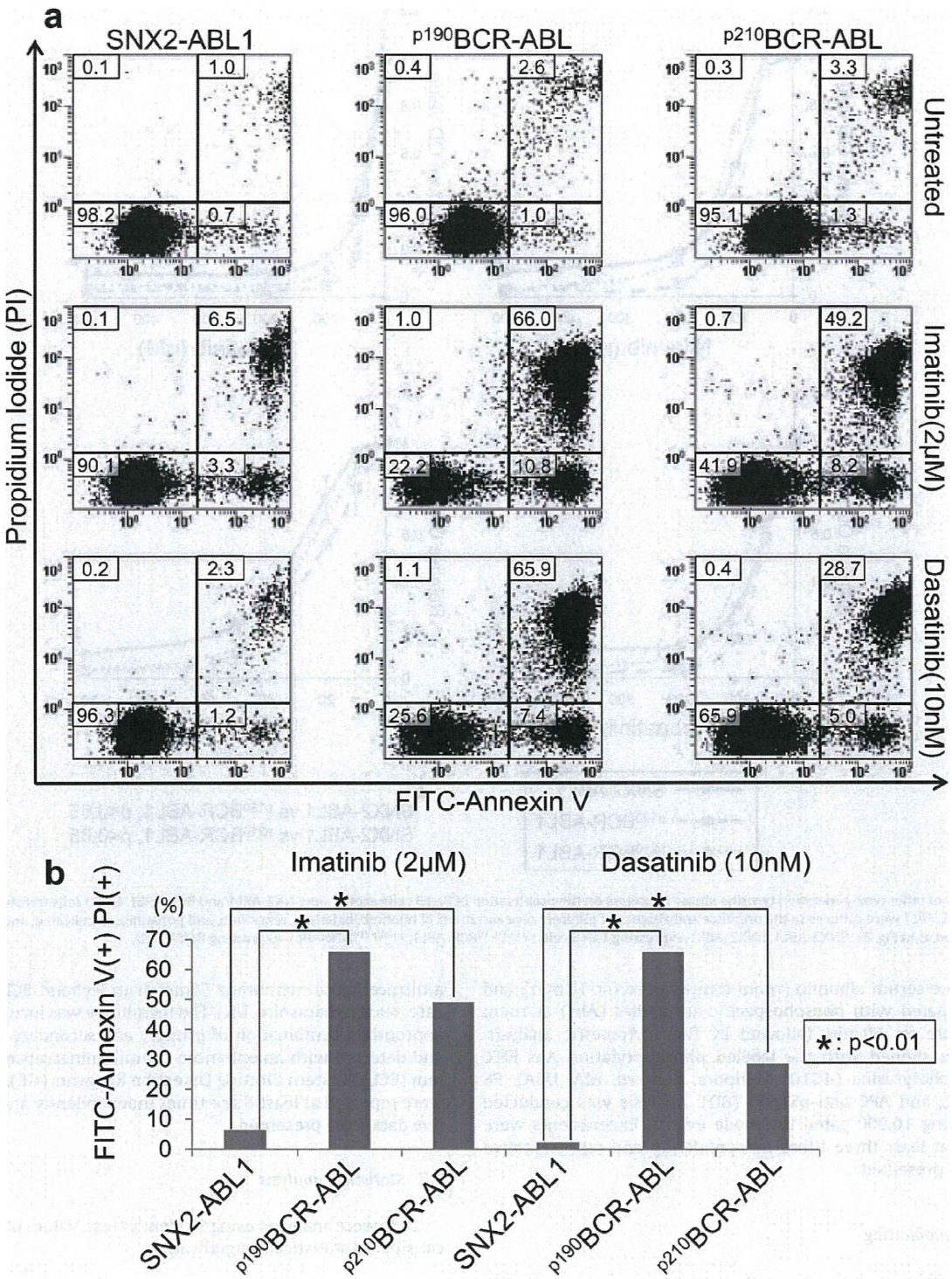

Fig. 4. Apoptosis induction mediated by imatinib and dasatinib in Ba/F3 cells expressing SNX2-ABL1 and BCR-ABL1. Ba/F3 transfectants were treated with 2 μM and 10 nM of imatinib and dasatinib, respectively, for 24 h, and the frequency of apoptotic cells was examined with the annexin-V-Propidium iodide (PI) staining. Experiments were performed in triplicate, and a typical cytogram (a) and the mean ± SEM of representative results of three independent experiments (b) are presented. SNX2-ABL1, SNX2-ABL1-expressing Ba/F3 cells; $^{p190/p210}$BCR-ABL1, $^{p190/p210}$BCR-ABL1-expressing Ba/F3 cells.

Analysis was conducted by collecting 10,000 gated list mode events. Each experiment was performed in triplicate and the mean ± SEM were indicated. Experiments were repeated three times independently and representative data were presented.

To detect phosphorylations of total intracellular proteins, CrkL, and STAT5, cells were fixed with cytofix/cytoperm buffer (BD Biosciences, San Jose, CA, USA, 37 °C for 10 min), permeabilized with methanol (final concentration 90%, 4 °C, 30 min), blocked with

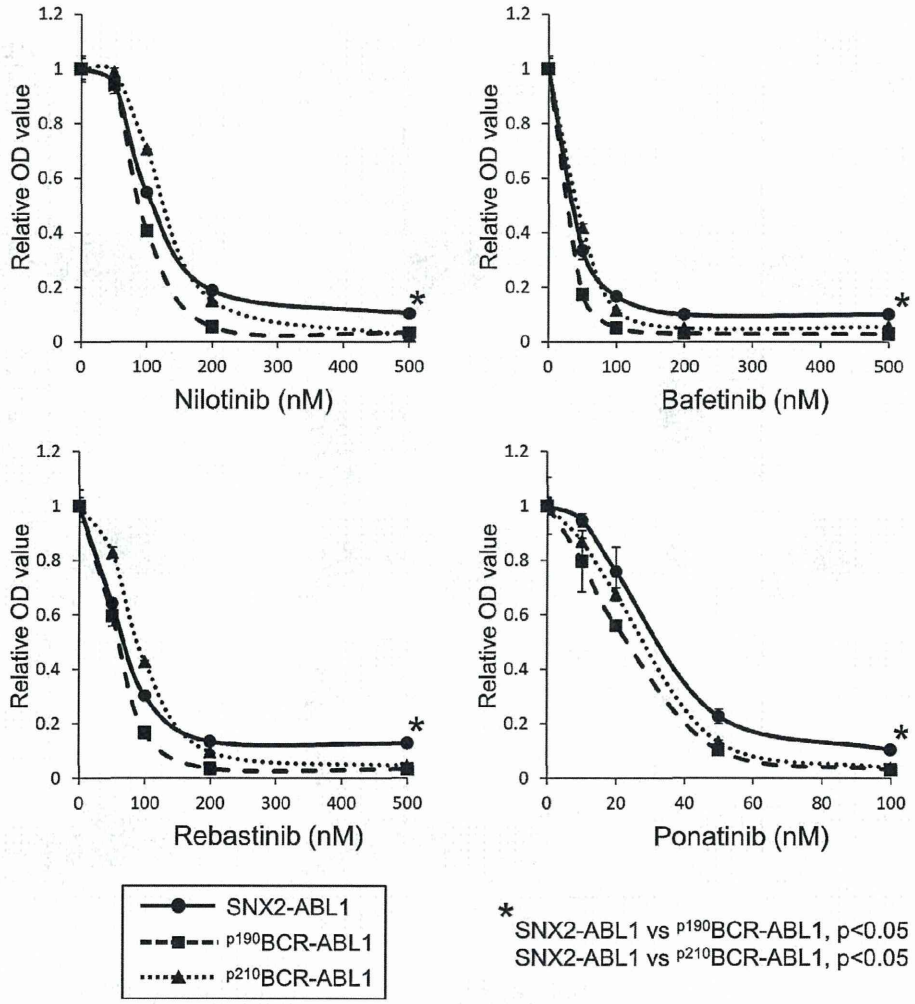

Fig. 5. Effect of other next-generation tyrosine kinase inhibitors on the proliferation of Ba/F3 cells expressing SNX2-ABL1 and BCR-ABL1. Ba/F3 cells transfected with *SNX2-ABL1* and *BCR-ABL1* were cultured in the presence and absence of different concentrations of nilotinib, bafetinib, rebastinib, and ponatinib, as indicated, and were examined and presented as in Fig. 3a. SNX2-ABL1, SNX2-ABL1-expressing Ba/F3 cells; $^{p190/p210}$BCR-ABL1, $^{p190/p210}$BCR-ABL1-expressing Ba/F3 cells.

0.5% bovine serum albumin (room temperature for 10 min), and then incubated with phosphospecific antibodies (Abs) at room temperature for 60 min, followed by flow cytometric analysis. Cells were stained with the labeled phosphorylation Abs FITC anti-phosphotyrosine (4G10; Millipore, Bedford, MA, USA), PE anti-pCrkL, and APC anti-pSTAT5 (BD). Analysis was conducted by collecting 10,000 gated list mode events. Experiments were repeated at least three times independently and representative data were presented.

2.6. Immunoblotting

Immunoblot analysis was performed as described previously [40] using the following Abs: anti-SNX2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-BCR, anti-pERK1/2 (p44/42 MAPK), anti-pCrkL (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphotyrosine (4G10; Millipore), anti-pSrc (Affinity BioReagents, ABR, Golden, CO, USA), anti-β-actin (Sigma–Aldrich) and horseradish peroxidise (HRP)-conjugated secondary Abs (anti-rabbit, anti-mouse or anti-goat polyclonal Abs) (Dako, Glostrup, Denmark). A 100-μg sample of each cell lysate was electrophoretically separated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Hybond-ECL; GE Healthcare, Buckinghamshire, UK). The membrane was incubated with an appropriate combination of primary and secondary Abs, washed, and detected with an enhanced chemiluminescence reagent system (ECL) Western Blotting Detection Reagents (GE). Experiments were repeated at least three times independently and representative data were presented.

2.7. Statistical analysis

Data were analyzed using Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Expression of SNX2-ABL1 induces IL-3-independent proliferation in Ba/F3 cells

To investigate the functional characteristics of SNX2-ABL1 protein in comparison with BCR-ABL1 protein, we introduced *SNX2-ABL1* as well as *BCR-ABL1* into murine Ba/F3 cells under the tetracycline-inducing system. Ba/F3 is an IL-3-dependent

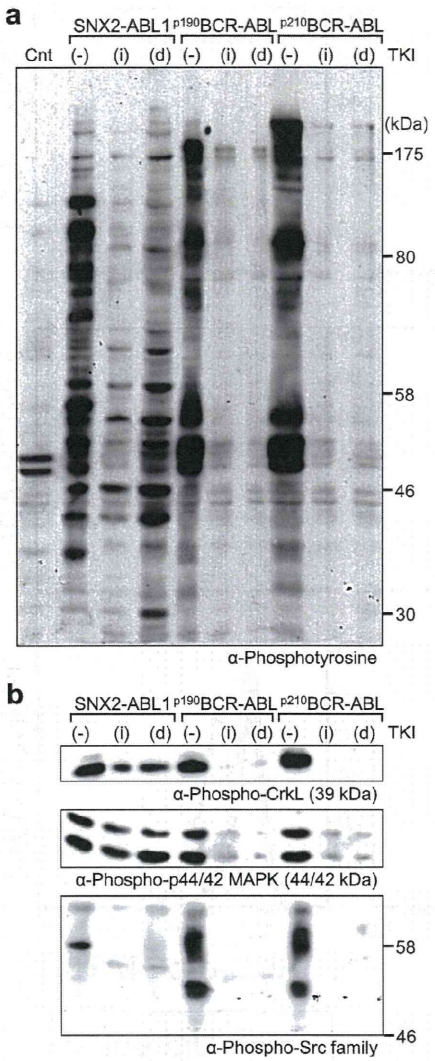

Fig. 6. Inhibitory effect of imatinib and dasatinib on protein phosphorylation in Ba/F3 transfectants detected by immunoblotting. Cell lysates were prepared from each Ba/F3 transfectant treated with and without 2 μM and 10 nM imatinib and dasatinib, respectively, for 10 min and immunoblot analysis was performed as in Fig. 1b using anti-phosphotyrosine (a) and phospho-specific (b) antibodies as indicated.

pro-B-cell line but can proliferate IL-3 independent manner by forced expression of some tyrosine kinase oncogenes such as *BCR-ABL1* and induction of factor independence of Ba/F3 cells has been considered to be closely related with transforming activity [33]. Since several variants of *BCR-ABL1* have been identified that differ in the breakpoint in *BCR* and are associated with distinct types of leukemia, we introduced *^{p210}BCR-ABL1* and *^{p190}BCR-ABL1* in Ba/F3 cells to compare with *SNX2-ABL1*. The *^{p210}BCR-ABL1* in which *BCR* exon 13 or 14 was fused in frame to *ABL1* exon 2 (e13a2 or e14a2 transcript), so called major *BCR-ABL1*, is generally associated with chronic myeloid leukemia, while *^{p190}BCR-ABL1* (e1a2 transcript, minor *BCR-ABL1*) is generally associated with ALL [4,5]. The schematic structures of SNX2-ABL1 and BCR-ABL1 kinases are presented in Fig. 1a. As shown in Fig. 1b, treatment with DOX effectively induced each ABL1-chimeric protein in the Ba/F3 transfectants, as assessed by immunoblotting.

Next, we analyzed the effect of the expression of ABL1-related chimeric molecules on the proliferation of Ba/F3 cells. As shown in Fig. 2, parental Ba/F3 and mock cells could only proliferate in the presence of IL-3 or conditioned medium from WEHI-3 cells. However, cells expressing SNX2-ABL1 as well as ^{p190}BCR-ABL1 and ^{p210}BCR-ABL1 molecules showed cell proliferation independent of the presence of IL-3 or WEHI-3 cell-conditioned medium. Their growth rate was equivalent to parental Ba/F3 cells growing in medium supplemented with IL-3. Conditioned medium prepared from the IL-3-independent cell lines expressing either SNX2-ABL1, ^{p190}BCR-ABL1, or ^{p210}BCR-ABL1 could not support the proliferation of parental Ba/F3 cells (data not shown).

3.2. Effect of TKIs on survival of Ba/F3 cells expressing ABL1-related chimeric molecules

We then investigated the selective activity of TKIs on SNX2-ABL1-expressing Ba/F3 cells in comparison with the BCR-ABL1-expressing cells. As shown in Fig. 3a, when ^{p190}BCR-ABL1- and ^{p210}BCR-ABL1-expressing Ba/F3 cells were exposed to imatinib and dasatinib, cell proliferation was significantly inhibited in a concentration-dependent manner, and the viable cell number was reduced to under 10% after 24-h incubation with imatinib at a concentration of 2 μM and dasatinib at a concentration of 10 nM. When SNX2-ABL1-expressing cells were exposed to imatinib and dasatinib, they showed relative resistance to these TKIs in compared with BCR-ABL1-expressing Ba/F3 cells. As shown in Fig. 3a, over 20 and 50% of the SNX2-ABL1-expressing cells still survived after 24-h incubation with imatinib at a concentration of 2 μM and dasatinib at a concentration of 10 nM, respectively. It is noteworthy that when cells were continuously cultured in the presence of TKIs, SNX2-ABL1-expressing cells began to proliferate again 48 h after treatment with dasatinib at a concentration of 10 nM (Fig. 3a and b). By employing the annexin V-Propidium iodide-binding assay, we further confirmed that the TKI-mediated reduction of the cell number was due to apoptosis (Fig. 4) and, again, SNX2-ABL1-expressing cells showed relative resistance to TKI-, especially dasatinib-, mediated apoptosis induction.

We also examined the effect of other next-generation TKIs, including nilotinib, bafetinib, rebastinib, and ponatinib. As shown in Fig. 5, each TKI effectively reduced numbers of Ba/F3 cells expressing ABL1-related chimeric molecules, whereas SNX2-ABL1-expressing cells showed slight resistance compared to BCR-ABL1-expressing Ba/F3 cells ($p < 0.05$).

3.3. SNX2-ABL1 induces the phosphorylation of intracellular proteins in Ba/F3 cells that are only partially inhibited by TKI treatment

Next, we assessed the molecular basis of the distinct sensitivity of SNX2-ABL1-expressing Ba/F3 cells against dasatinib-mediated cell death from that of BCR-ABL1-expressing Ba/F3 cells. As shown in (Fig. 6a), immunoblot analysis revealed a significant increase in the tyrosine-phosphorylation level of intracellular proteins in Ba/F3 cells after the induction of SNX2-ABL1. Therefore, we concluded that SNX2-ABL1 is activated and induces tyrosine-phosphorylation in a number of intracellular proteins in Ba/F3 cells, as in the case of BCR-ABL1. Interestingly, the tyrosine-phosphorylation pattern of the proteins, including Src family protein kinases, in SNX2-ABL1-expressing Ba/F3 cells was different from those of $^{p190/p210}$BCR-ABL1-expressing Ba/F3 cells (Fig. 6a and b).

Regarding the effect of imatinib and dasatinib on the level of protein tyrosine-phosphorylation, only partial inhibition of the tyrosine-phosphorylation of proteins was observed in the SNX2-ABL1-expressing Ba/F3 cells, while the tyrosine-phosphorylation of proteins was significantly reduced in BCR-ABL1-expressing Ba/F3 cells, as assessed by immunoblotting (Fig. 6a). We further examined the effect of TKI-treatment on phosphorylation levels in individual

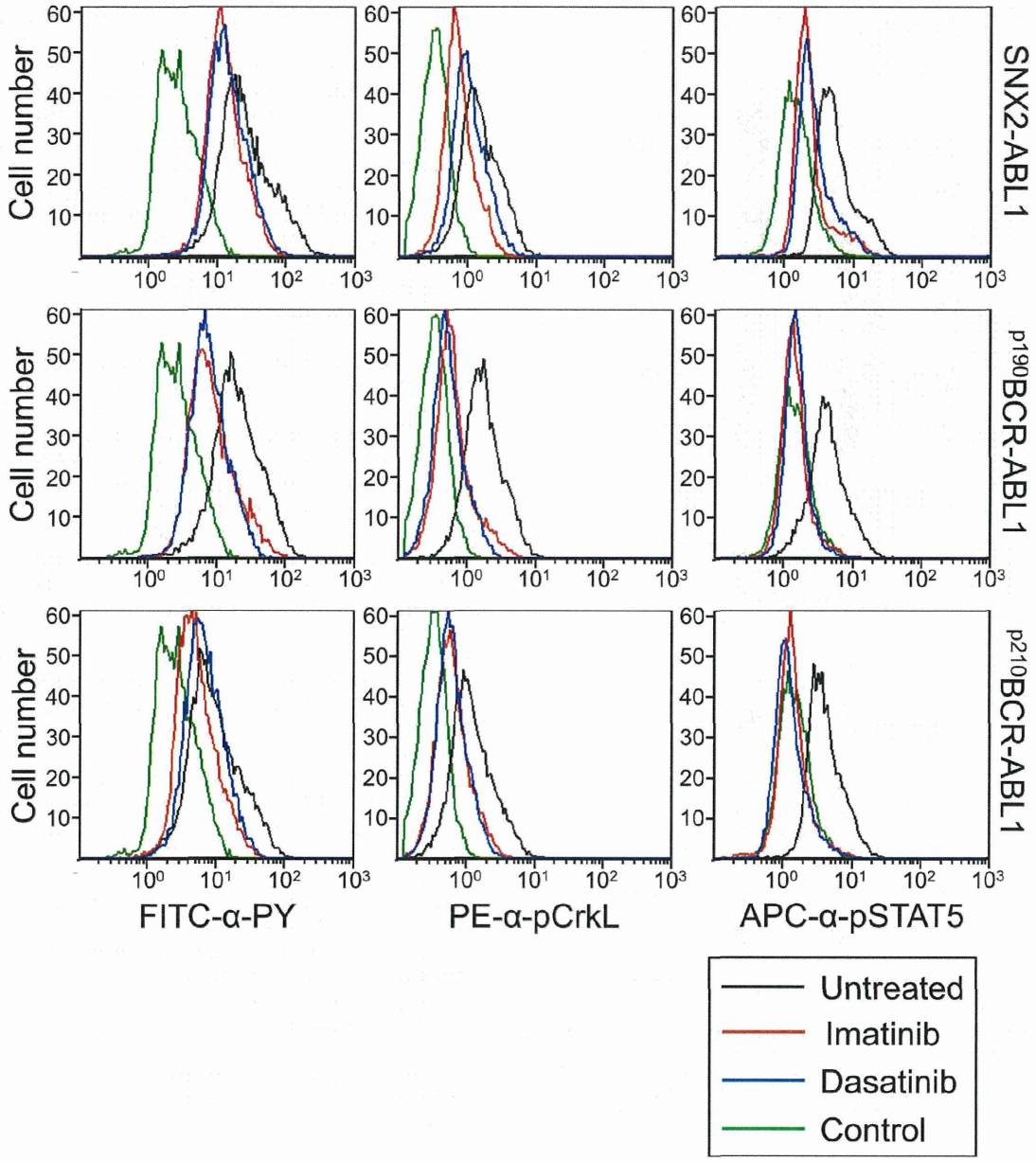

Fig. 7. Inhibitory effect of imatinib and dasatinib on protein phosphorylation in Ba/F3 transfectants detected by flow cytometry. Ba/F3 transfectants were treated as in Fig. 6, and the phosphotyrosine (4G10), phosphorylation states of CrkL and STAT5 were examined by flow cytometry.

downstream molecules for ABL1 kinase by immunoblot analysis, and observed that the phosphorylation of CrkL, $^{p44/42}$MAP kinase, and Src family protein kinases were significantly reduced in BCR-ABL1-expressing Ba/F3 cells, whereas CrkL and $^{p44/42}$MAP kinase were still sustained to a certain extent in SNX2-ABL1-expressing Ba/F3 cells (Fig. 6b).

We further confirmed by flow cytometric analysis that the levels of tyrosine-phosphorylation of intracellular proteins as well as CrkL and STAT5 were significantly reduced in BCR-ABL1-expressing Ba/F3 cells upon TKI-treatment, while they maintained a phosphorylated state even after treatment with TKIs in SNX2-ABL1-expressing Ba/F3 cells (Fig. 7).

4. Discussion

The expression of *SNX2-ABL1* induces the ability to proliferate in the absence of exogenous IL-3 in Ba/F3 cells, indicating the transforming potential [33] of *SNX2-ABL1* fusion, similarly to the case of *BCR-ABL1*. Including BCR, the partner protein composing the N-terminal part of ABL1-related chimera often includes a coiled-coil or helix-loop-helix domain that allows oligomerization of the protein required for the constitutive activation of ABL1 tyrosine kinase [4]. In the case of SNX2, however, no specific domain structure is retained in the SNX2-ABL1 fusion protein (primary amino acid positions 1–130, Fig. 1a) and, thus, it is debatable whether or not

remaining part of the SNX2 protein contributes to oligomerization of the fusion protein.

In contrast, the kinase domain of ABL1 is fully retained in the SNX2-ABL1 fusion protein (Fig. 1a). Although most *ABL1* fusions reported to date result in the partner gene fusing to *ABL1* exons 2 [27–29], *SNX2* exon 3 was fused in frame to *ABL1* exon 4 in both our [37] and a previously reported case [26]. As we presented in Fig. 1a, such a fusion lacks the amino acids to form an intact Src homology 2 (SH2) domain and Src homology 3 (SH3) domain, respectively [30,31]. The activity of ABL1 protein is negatively regulated by its SH3 domain, and deletion of the SH3 domain makes ABL1 oncogenic [41]. In addition, the interactions between SH3 and SH2 domains provide a regulatory clamp that allosterically holds the kinase domain in a tightly downregulated state [42]. These findings suggest that a deficit of both SH2 and SH3 domains contribute to the constitutive activation of SNX2-ABL1 tyrosine kinase.

More importantly, our data also demonstrate that SNX2-ABL1-expressing Ba/F3 cells show distinct sensitivity for TKIs, especially dasatinib resistance, in comparison with BCR-ABL1-expressing Ba/F3 cells, indicating the ineffectiveness of dasatinib as a therapeutic reagent for patients with *SNX2-ABL1*+ ALL. As we described above, our patient with *SNX2-ABL1*+ ALL poorly responded to dasatinib but partially responded to imatinib [37], and, thus, his clinical course corresponds well to our experimental results in the present study.

Besides our patient, *SNX2-ABL1* has been reported only once in an adult patient with BCP-ALL in the literature [26], and the patient also experienced early relapse after the initial chemotherapy, transiently responded to treatment with imatinib, and died despite salvage chemotherapies. The involvement of exon 4 of the *ABL1* gene was also reported in an *RCSD1-ABL1* fusion gene caused by a t(1;9)(q24;q34) translocation [31], and a case of BCP-ALL associated with a *RCSD1-ABL1* fusion gene treated by imatinib and dasatinib, combined with dexamethasone, showed transient clinical effects, whereas leukemic cells rapidly became refractory to the treatment [32]. Therefore, the deficit of SH3 and SH2 domains in ABL1-related fusion molecules is possibly related to TKI resistance too.

As we discussed above, the absence of both SH3 and SH2 domains may result in the loss of a negative regulatory element for the kinase domain and, thus, induce more aggressive activation of ABL1 kinases with distinct kinase domain conformation related to TKI resistance. Alternatively, the deficit of SH3 and SH2 domains may introduce a distinct downstream signaling of ABL1 kinases and lead to the activation of a different set of mediators or substrates and our data suggesting a distinct tyrosine phosphorylation pattern of cellular proteins mediated by SNX2-ABL1 from that by BCR-ABL1 should support this notion. For example, it has been reported that oncogenic Src family kinases (SFKs) are activated through SH3 and SH2 domain-mediated direct interaction with BCR-ABL1 [43] and interact collaboratively in BCR-ABL1-induced leukemogenesis [44]. It was also reported that SFKs, including Hck, Lyn, and Fyn, phosphorylate multiple tyrosine residues within the SH3 and SH2 domains of BCR-ABL1, and that these phosphorylations are required for the full oncogenicity of BCR-ABL1 [45]. Therefore, SNX2-ABL1 with a deficit of both SH3 and SH2 domains should utilize alternate signaling pathways to achieve full oncogenicity. The fact that dasatinib is a multi-targeted inhibitor and effective for SFKs as well as BCR-ABL1 [16] may explain, at least in a part, dasatinib resistance of SNX2-ABL1.

Concerning resistance to dasatinib in Ph+ leukemia patients, the contribution of mutations in the *ABL1* gene, especially T315I and F317L, has been reported [46]. However, since our structures of *BCR-ABL1* and *SNX2-ABL1* do not have any mutations in the *ABL1* kinase gene, this is not the case for resistance to dasatinib in *SNX2-ABL1*+ ALL. Besides the mutations in the *ABL1* gene, a number of BCR-ABL1-independent mechanisms are known to confer resistance to TKIs, including the overexpression of a P-glycoprotein efflux pump and low expression and activity of organic cation transporter 1 (OCT1) [47–49]. Therefore, it might be worth investigating the involvement of these molecules in the distinct sensitivity of SNX2-ABL1 toward tyrosine kinase inhibitors from that of BCR-ABL1.

As we presented in this study, SNX2-ABL1 exhibited a poorer response to dasatinib but a relatively favorable response to nilotinib. Nilotinib has a similar structure to imatinib and shares its binding mode and high specificity, whereas, as we described above, dasatinib differs from imatinib in its chemical structure, binding mode, and pharmacokinetic properties, having a rather broad specificity, and it inhibits other kinases [50–52]. Therefore, the high specificity for ABL1 kinase should be important for the therapeutic use of TKIs in patients with *SNX2-ABL1*+ ALL.

In conclusion, SNX2-ABL1 has a transforming potential, but exhibits distinct sensitivity to TKIs in comparison with BCR-ABL1. Although further investigation is needed to assess the precise mechanisms, both clinical and experimental findings concordantly indicate the possibility of resistance of SNX2-ABL1 kinase to TKIs, especially dasatinib, and, thus, other TKIs with a more selective effect against this chimeric kinase should be used for treatment.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

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DNA Methylation Profile Distinguishes Clear Cell Sarcoma of the Kidney from Other Pediatric Renal Tumors

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Abstract

A number of specific, distinct neoplastic entities occur in the pediatric kidney, including Wilms' tumor, clear cell sarcoma of the kidney (CCSK), congenital mesoblastic nephroma (CMN), rhabdoid tumor of the kidney (RTK), and the Ewing's sarcoma family of tumors (ESFT). By employing DNA methylation profiling using Illumina Infinium HumanMethylation27, we analyzed the epigenetic characteristics of the sarcomas including CCSK, RTK, and ESFT in comparison with those of the non-neoplastic kidney (NK), and these tumors exhibited distinct DNA methylation profiles in a tumor-type-specific manner. CCSK is the most frequently hypermethylated, but least frequently hypomethylated, at CpG sites among these sarcomas, and exhibited 490 hypermethylated and 46 hypomethylated CpG sites in compared with NK. We further validated the results by MassARRAY, and revealed that a combination of four genes was sufficient for the DNA methylation profile-based differentiation of these tumors by clustering analysis. Furthermore, *THBS1* CpG sites were found to be specifically hypermethylated in CCSK and, thus, the DNA methylation status of these *THBS1* sites alone was sufficient for the distinction of CCSK from other pediatric renal tumors, including Wilms' tumor and CMN. Moreover, combined bisulfite restriction analysis could be applied for the detection of hypermethylation of a *THBS1* CpG site. Besides the biological significance in the pathogenesis, the DNA methylation profile should be useful for the differential diagnosis of pediatric renal tumors.

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Introduction

In the pediatric population, the types of renal tumor are entirely different from those occurring in adults. It is estimated that 85% of pediatric renal malignancies comprise nephroblastoma, 5% congenital mesoblastic nephroma (CMN), 4% clear cell sarcoma of the kidney (CCSK), and 2% rhabdoid tumor of the kidney (RTK) [1], and these 4 major entities account for 96% of the total. The remaining 4% tend to occur in older children and include miscellaneous tumors, such as the Ewing's sarcoma family of tumors (ESFT). Nephroblastoma is malignant but still a relatively favorable tumor prognostically, being derived from nephrogenic blastemal cells that can show divergent differentiation. CMN is a kind of fibroblastic sarcoma of infancy and characterized by a specific chromosomal translocation, t(12;15)(p13;q25), which results in the fusion of *ETV6* and *NTRK3* genes [2]. On the other hand, CCSK is a relatively unfavorable tumor prognostically, being composed of clear mesenchymal cells with a characteristic vascular pattern [3]. RTK is a highly aggressive tumor occurring in young children, has a dismal outcome, and is characterized by pathological rhabdoid features and molecular biallelic inactivation of the *SMARCB1* (*hSNF5/INI1*) gene [4–6].

Since pediatric renal tumors are diverse neoplastic entities, as described above, and require different therapeutic strategies, rapid and accurate diagnosis is crucial for adequate treatment. However, all those tumors are composed of small-to-medium-sized, round, oval, or spindle-shaped undifferentiated or immature cells, and often deceptively mimic each other, making the diagnosis difficult [7]. In RTK and CMN, molecular markers, i.e., loss of SMARCB1 expression and *ETV6-NTRK3* fusion, respectively, are useful for an ancillary diagnosis, whereas the diagnosis of nephroblastoma and CCSK is exclusively based on histologic features. Although numerous studies have been done, immunohistochemical features or recurrent genetic changes that can reliably distinguish CCSKs from other pediatric renal tumors have not identified [3,8]. Therefore, the identification of molecular signatures that can distinguish CCSK from other renal tumors should be useful and provide diagnostic confidence and accuracy.

Alterations of DNA methylation have been well documented as an important peculiarity of cancer cells [9,10], and two patterns of