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Gain-of-function *c-CBL* mutations associated with uniparental disomy of 11q in myeloid neoplasms

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***c-CBL (CBL)* encodes a multifunctional protein engaged in the regulation of intracellular signaling pathways.[1,2] It was first identified as a cellular counterpart of the viral oncogene, *v-CBL*, that causes murine lymphoma.[3,4] Although no genetic evidence existed suggesting its role in human carcinogenesis, the recent discovery of c-*CBL* mutations in myeloid cancers has unveiled a unique oncogenic mechanism mediated by gain-of-function of a mutated tumor suppressor, closely associated with allelic conversion of 11q arms.[5-9] In this review, we summarize our current knowledge about *c-CBL* mutations and discuss the molecular mechanisms of their gain-of-function.**

Myeloproliferative Neoplasms and Related Disorders

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of blood cancers, characterized by clonal hematopoiesis that causes excessive production of one or more components of mature blood cells with hypercellular bone marrow and extramedullary hematopoiesis.[10] Some patients also show abnormalities in cell morphology and differentiation with dysplastic bone marrow, and are classified into myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in the new World Health Organization (WHO) classification.[11] A genetic hallmark of MPN and MDS/MPN is frequent mutations of genes on signal transduction pathways, which have been causally linked to hypersensitivity of neoplastic progenitors to growth factors and cytokines.[10] A notable example is *JAK2 V617F* mutations found in most cases of polycythemia vera (PV), a form of MPNs that is characterized by overproduction of mature erythrocytes together with other blood components.[12-14]

JAK2 Mutations in MPNs

These mutants encode constitutive active kinases that transmit signals from erythropoietin receptor, and induce a hypersensitive proliferative response to erythropoietin.[12] Of particular interest about *JAK2* mutations in PV is the presence of one or more subclones showing acquired uniparental disomy (aUPD) involving the 9p arm that leads to homozygous *JAK2* mutations ($JAK2^{mut/mut}$) by allelic conversion (**Fig. 1**).[15] One of the initial discoveries of *JAK2* mutations relied on the detailed mapping of loss of heterozygosity (LOH) caused by aUPD in 9p.[13] The consequence of 9p-aUPD is loss of wild type *JAK2* and duplication of mutated *JAK2*, but the latter seems to be more important for the clonal selection of UPD clones, because mutated *JAK2* is duplicated without loss of wild-type allele in 9p trisomy in some cases.[16] Similarly gain-of-function mutations of *cMPL* are

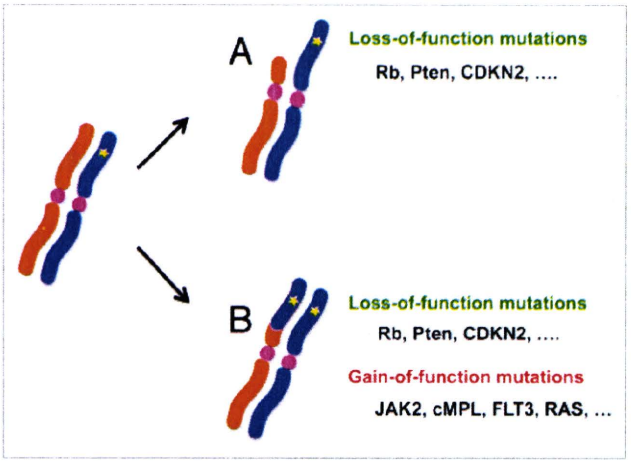

Figure 1. In cancer cells, LOH is frequently associated with a mutated tumor suppressor locus, in which a normal copy of the tumor suppressor is lost by simple allelic deletion (A), or replaced by the mutated copy through allelic conversion that leads to copy number neutral LOH or aUPD (B). In either case, the common consequence is biallelic loss-of-function of the tumor suppressor. In addition, LOH caused by aUPD is also implicated in the common mechanism of homozygous mutations of proto-oncogenes. A number of gain-of-function oncogenic mutations found in aUPD regions have been shown to exist in a homozygous state, including mutations of *JAK2* (9pUPD), *MPL* (1pUPD), *NRAS* (1pUPD), *KRAS* (12pUPD), *BRAF* and *FLT3* (13qUPD). The clonal outgrowth of aUPD-positive clones indicates that two copies of mutations confer a growth advantage to aUPD positive cells through their gain-of-function.

frequently found in primary myelofibrosis in close association with 1p-aUPD.[17] Thus, aUPD, or copy number neutral LOH, is associated not only with biallelic loss-of-function of classical tumor suppressor genes in the Knudson's paradigm,[18] but also with gain-of-function of proto-oncogenes. Moreover, genome-wide analysis of genetic imbalances in a variety of myeloid neoplasms revealed that aUPD is another genetic feature of MPNs, where 42% of chronic myelomonocytic leukemia (CMML) cases had one or more regions of aUPD and were grouped into several discrete clusters, which may or may not harbor mutations of known cancer related genes.[9] Among these one of the most prominent is the cluster that is defined by 11q-aUPD, from which mutated *c-CBL* proto-oncogene was identified.[9]

c-CBL Mutations in MDS/MPNs

Although *c-CBL* mutations have been reported in a variety of myeloid neoplasms including acute myeloid leukemia, myelodysplastic syndromes, as well as classical myeloproliferative disorders, the majority of *c-CBL*-mutated cases are MDS/MPN, including CMML (~15%), juvenile myelomonocytic leukemia (JMML) (~17%), and atypical chronic myeloid leukemia (~5%).[5-9,19,20] In most cases, *c-CBL* mutations are associated with 11q-aUPD involving *c-CBL* locus, which converts these mutations into a homozygous state. Loss of wild-type *c-CBL* is rarely caused by chromosomal deletion.[6,7,9] *c-CBL* mutations exclusively occur independent of *RAS* and *PTPN11* in CMML and JMML.[8,9] Notably, *c-CBL* mutations have a germline origin in some JMML cases.[8] Approximately half of the *CBL* mutations in JMML cases involve Y371, while mutations are widely distributed within linker/RING finger domain in other neoplasms. *c-CBL* mutants strongly transform fibroblasts and enhance proliferation of hematopoietic progenitors in methylcellulose culture.[9] These genetic and functional observations indicate that mutant *c-CBL* may have some gain-of-function, which promotes clonal evolution, especially of aUPD-positive clones carrying two copies of the mutations.

c-CBL as a Tumor Suppressor Gene

c-CBL proto-oncogene is a cellular homologue of a viral oncogene, *v-CBL*, isolated from the Casitas-NS-lymphoma virus that induces murine lymphoma.[3,4] Together with other two homologues CBL-b and CBL-c, it comprises the CBL family of proteins. All c-CBL proteins have an N-terminal domain for binding to phosphorylated tyrosine kinases (TKB domain) connected through a linker sequence to the RING finger, but CBL-c lacks most of the C-terminal domains shared by c-CBL and CBL-b (**Fig. 2A**). While c-CBL has multivalent molecular functions in signal transduction and cytoskeletal regulation, the most intensively studied-function is its role in negative regulation of receptor tyrosine kinase (RTK) signalings, which depends on the E3 ubquitin ligase activity of this molecule.[1,21] After RTKs are phosphorylated on cytokine stimulation, c-CBL binds to the phosphorylated RTKs through the TKB domain, and mono-ubiquitinates these RTKs at multiple sites in concert with the E2 conjugating enzyme, which is followed by internalization and degradation/recycling of the phosphorylated RTKs.[21] Thus, c-CBL prevents excessive RTK signaling after cytokine/growth factor stimulation and potentially acts as a tumor suppressor. *c-CBL*$^{-/-}$ mice have an enlarged thymus, splenomegaly with extramedullary hematopoiesis.[22,23] In these mice, hematopoietic progenitor pools are expanded,[9,24] and their hematopoietic progenitors exhibit hypersensitive proliferative responses to cytokine stimulations. When introduced into *BCR/ABL* transgenic mice, a *c-CBL*$^{-/-}$ allele accelerates blastic crisis.[9] Moreover, *c-CBL*$^{-/-}$ mice developed invasive cancer spontaneously (in preparation), further supporting that *c-CBL* has tumor suppressor functions.

Gain of Function of CBL Mutants

How can we reconcile with the tumor suppressor functions of c-CBL on the one hand, and the oncogenic properties of c-CBL mutants on the other? A simple explanation would be an inhibition of tumor suppressor function of wild type c-CBL by mutant c-CBL. Most *c-CBL* mutations in MPNs occur within the linker/RING finger domains, through which c-CBL binds E2 conjugating enzymes, and thus are expected to compromise the E3 ligase activity of the molecule.

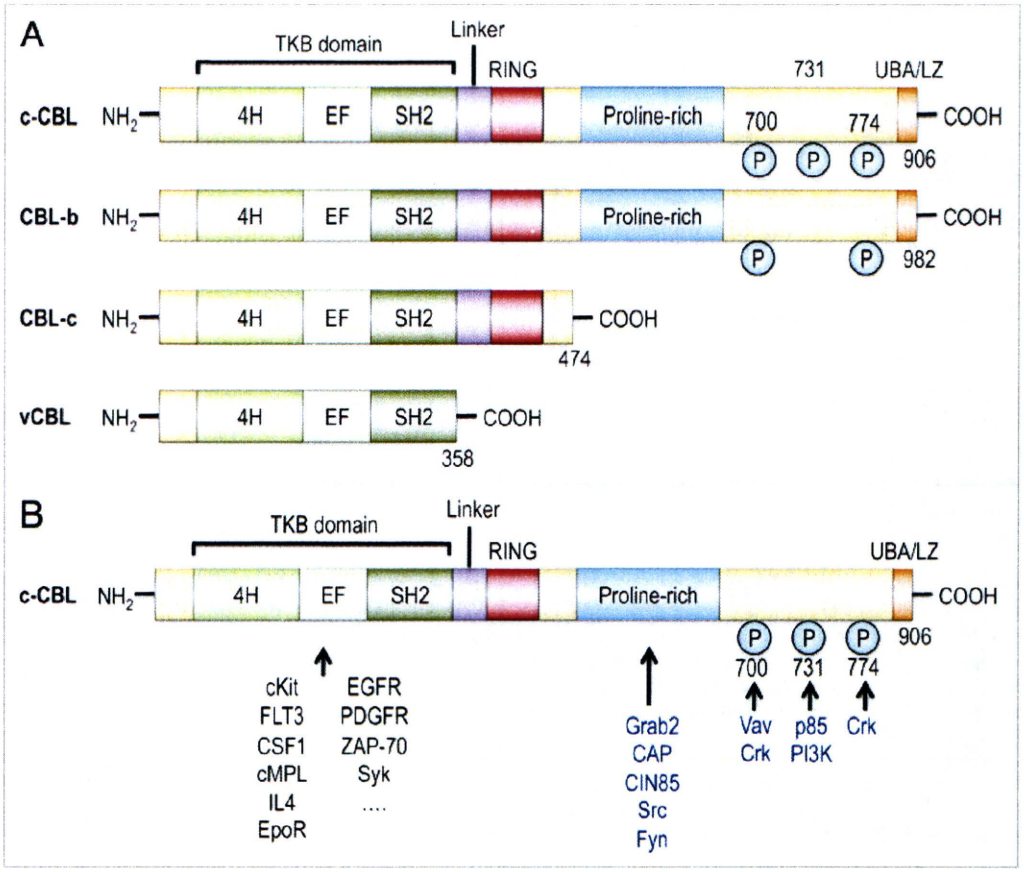

Figure 2. (A) Structure of CBL family proteins. CBL family proteins in mammals have highly conserved domains, where an N-terminal TKB domain, consisting of a four-helix bundle (4H), a Ca^{2+}-binding EF (EF), and a src-homology (SH2) domains, is connected to a RING finger domain via a linker. c-CBL and CBL-b, but not CBL-c, have a proline-rich and other C-terminal components that end with a ubiquitin-associated and leucine zipper (UBA/LZ) domain. Their viral form, v-CBL, is truncated just after its SH2 domain. (B) CBL family proteins interact with a number of signal transducing molecules. Through their TKB domain, CBL family proteins target phosphrylated tyrosine kinases, including growth factor receptors and cytokine receptors, as well as, non-receptor tyrosine kinases. Ubiquitin conjugating enzymes have contact with CBL proteins via the linker/RING finger domain, which is central to the E3 ubiquitin ligase activity. The proline-rich domain provides a binding site for SH3 domains of Grab2, CAP and Src-family kinases. The C-terminal portion contains three tyrosine residues, Y700, Y731 and Y774, which are the major phosphorylated tyrosines, and which bind to the p85 subunit of PI3 kinase (Y731), Vav (Y700) and Crk proteins (Y700 and Y774).

In fact, when expressed in fibroblasts, tumor-derived linker and RING finger mutants show severely compromised E3 ubiquitin ligase activity.[9,20] Moreover, two linker mutants (Q367P and Y371S) have been shown to inhibit the activity of wild-type c-CBL protein, although they do not make direct contact with E2 enzymes but with the TKB domain.[25] As expected from the inhibitory action of these mutants with regard to E3 ubiquitin ligase activity, transduction of the latter mutants into NIH3T3 or hematopoietic cells lead to prolonged activation of tyrosine kinases after stimulation with a variety of cytokines and growth factors, including epidermal growth factor, stem cell factor (SCF), Interleukin 3 (IL3), thrombopoietin, and FLT3 ligand.[9,20] Given the diverse spectrum of kinase targets of CBL, the enhanced sensitivity of these cells to a variety of cytokines is well expected.

Although these experimental data support a dominant negative mechanism of mutant *c-CBL*, a simple dominant negative model is defied by an experiment, in which mutant *c-CBL* was transduced into *c-CBL*$^{-/-}$ hematopoietic progenitors. Lin$^-$Sca1$^+$cKit$^+$ (LSK) hematopoietic progenitors from *c-CBL*$^{-/-}$ mice showed enhanced survival or proliferative responses after stimulation with a variety of cytokines, including SCF, IL3, or thrombopoietin, as compared to those from *c-CBL*$^{+/+}$ mice. However, transduction of mutant *c-CBL* into *c-CBL*$^{-/-}$ progenitors dramatically augmented the responses to these cytokines and also to FLT3 ligand, while the effect of mutant *c-CBL*-transduction into *c-CBL*$^{+/+}$ progenitors was unremarkable even as compared to mock-transduced *CBL*$^{-/-}$ progenitors.[9] The augmented sensitivity to these cytokines in *c-CBL*$^{-/-}$ cells was nothing to do with the inhibition of c-CBL functions, and thus is considered to represent a true gain-of-function of the mutant c-CBL. The gain-of-function nature of *c-CBL* mutations is also predicted

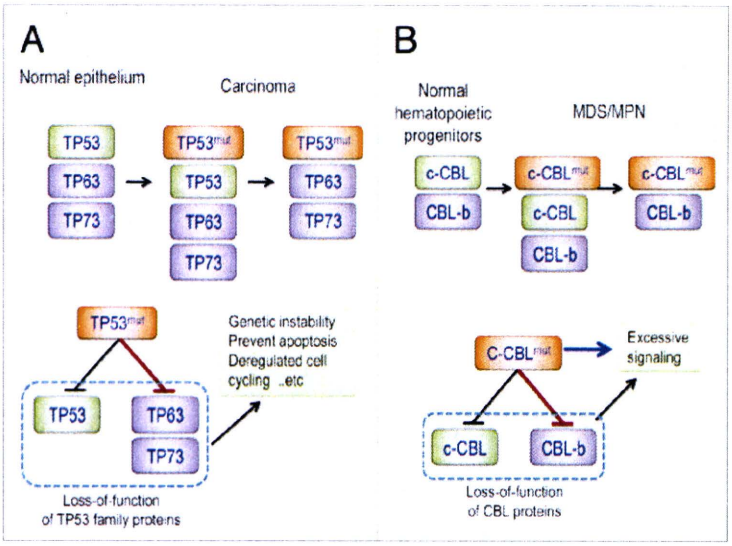

Figure 3. Possible mechanisms of gain-of-function of mutated TP53 and c-CBL. The gain-of-function of TP53 mutants is associated with their potential to induce carcinoma in mice as well as in human, which is considered to be mediated by inhibition of TP63 and TP73. TP53-deficient mice frequently develop sarcomas and lymphomas but only rarely carcinomas, which are thought to be suppressed by TP53 homologues, TP63 and TP73, in epithelial tissues, in the face of loss of TP53. Mutant TP53 inhibits tumor suppressor functions of TP63 and TP73, and compromises TP53-like activity. Similarly, the gain-of-function of CBL mutants found in MDS/MPN may be explained by the inhibition of CBL-b (red arrow), which would result in more profound defects in negative regulation of tyrosine kinase signaling compared to simple loss of c-CBL. On the other hand, c-CBL is thought to have positive regulatory functions that are not directly related to the E3 ubiquitin ligase activity and could be the source of the gain-of-function of c-CBL mutants (blue arrow).

from the fact that in myeloid neoplasms, 11qLOH is caused by aUPD in most cases and rarely accompanies 11q deletion, although in this case the target gene has tumor suppressor functions. Interestingly, the effect of the gain-of-function effect largely disappears by introducing wild type *c-CBL* or in the presence of the wild-type *c-CBL* allele,[9] which might explain the observation that the wild type *c-CBL* allele was lost in most MDS/MPN cases with *c-CBL* mutations as a result of allelic conversion or aUPD.

Origin of the Gain-of-Function of Mutant CBL

The exact mechanism through which mutant c-CBL acquires oncogenic functions even in *c-CBL*$^{-/-}$ cells is still elusive. Because the gain-of-function of mutant c-CBL is largely neutralized by the presence of wild type c-CBL, one possibility is that it could be mediated by the inhibition of some 'CBL-like' activity still present in *c-CBL*$^{-/-}$ cells, most likely CBL-b. Both c-CBL and CBL-b are expressed in immature hematopoietic progenitors, and c-CBL mutant inhibits E3 ubiquitin ligase activity of both c-CBL proteins.[9,26] Although *c-CBL/CBL-b*-double knockout mice are embryonic lethal, conditional double knockout in T cells shows hypersensitive to anti-CD3 stimulations and prolonged TCR-signaling, as compared to *c-CBL* or *CBL-b* single null T cells.[27] This reminds us of the gain-of-function of mutated TP53, which explains the difference in the phenotypes between *TP53*$^{-/-}$ and *TP53*$^{mut/-}$ mice. *TP53*$^{-/-}$ mice develop tumors at a high frequency, but they are mostly sarcomas or lymphomas and development of carcinoma is very rare, whereas *TP53*$^{mut/-}$ mice also develop carcinoma in various organs. Thus, TP53 mutant has more than null functions, which are thought to be mediated by the inhibition of its homologues, TP63 and TP73, expressed in epithelial tissues (**Fig. 3**).[28,29] Like c-CBL, TP53 tumor suppressor gene was first identified as an oncogene through its mutated, oncogenic forms in cancer cells. On the other hand, the model of gain-of-function mediated through CBL-b inhibition fails to explain why *CBL-b* mutations are extremely rare in CMML. According to this model, essentially no difference would be expected between the mutations of *c-CBL* and *CBL-b*, as long as in either case, compromised E3 ubiquitin ligase activity would result. The linker-RING finger mutants of c-CBL would be expected also to be able to inhibit E3 ubiquitin ligase activity of the wild-type c-CBL.

Another, but not necessarily exclusive, explanation of the gain-of-function of mutant c-CBL would be related to positive roles of c-CBL as a signal transducer rather than an attenuator (**Figs. 3A and 4**). c-CBL not only binds to a number of phosphorylated tyrosine kinases through its TKB domain, which is indispensable for the negative regulation of these kinases, but also interacts with more than 150 different proteins through a number of C-terminal domains and residues, and acts as a multi-domain adaptor protein, involved in signal transduction (**Fig. 2B**).[2] When recruited to phosphorylated tyrosine kinases, c-CBL is also phosphorylated at multiple tyrosine residues, and provides docking sites for the SH2 domains of Vav (pY700),[30] CrkL (pY700 and pY774)[31-34] and the p85 subunit of PI3 kinase (Y731).[35-37] c-CBL also binds to Grab2,[38-40] CAP,[41] and Src family tyrosine kinases[36] through the proline-rich domain. Several lines of evidence suggest that c-CBL positively transmits signals through these interactions. For example, c-CBL promotes cell survival and proliferation, depending on the PI3 kinase pathway,[42,43] and also enhances activation of MAP kinases after stimulation of Met tyrosine kinase.[44] c-CBL is also a key substrate/effector of Src kinase, which plays a central role in bone resorption and osteoclast migration.[45,46] It also is involved in cytoskeletal rearrangements through activation of Rac1 and Cdc42, and R-Ras.[47,48] Normally, mediated by its E3 ligase activity, kinase-bound phosphorylated c-CBL rapidly undergoes degradation,[26] by which positive signaling should be terminated. Thus, once linker/RING finger mutations abolish the E3 ligase activity of c-CBL, the consequence would be prolonged signaling due not only to loss of negative

regulation of tyrosine kinase, but also to enhanced positive regulatory functions, which should appear as gain-of-function (Fig. 4).

In contrast to the CBL-b inhibition model, the uni-laterality of c-CBL mutations could be more easily explained, because c-CBL and CBL-b have distinct biological functions, as clearly shown by the phenotypes of *c-CBL*$^{-/-}$ and *CBL-b*$^{-/-}$ mice.[22,23,49,50] For example, CBL-b lacks one of the major phosphorylated tyrosines, Y731, that provides a docking site for the p85 subunit of PI3 kinase. Although the exact molecular basis for the distinct functions between both CBL proteins remains to be elucidated, c-CBL-specific positive regulatory function in immature hematopoietic progenitors may be important for the pathogenesis of myeloid neoplasms.

Conclusion

Allelic conversion leading to aUPD is an important genetic mechanism of clonal evolution in the pathogenesis of MPN, and associated not only with loss-of-function of tumor suppressor genes, but also with gain-of-function mutations of proto-oncogenes. Homozygous *c-CBL* mutations that characterize a subset of MDS/MPD carrying 11q-aUPD, represent a unique example of gain-of-function mutations of tumor suppressor/proto-oncogene. These linker/RING finger mutations convert c-CBL, which otherwise act as a tumor suppressor, to a gain-of-function oncogenic protein. Although its exact molecular mechanism is still unknown, the gain-of-function of oncogenic c-CBL mutants seems to be related to disintegration of negative and positive regulatory machineries of normal c-CBL protein. Detailed analysis of the oncogenic mechanisms of c-CBL mutants is warranted, which should shed light on a novel aspect of physiological function of c-CBL. Considering their expression and functions in a broad spectrum of tissues, *CBL* family genes may be mutated in other human cancers.

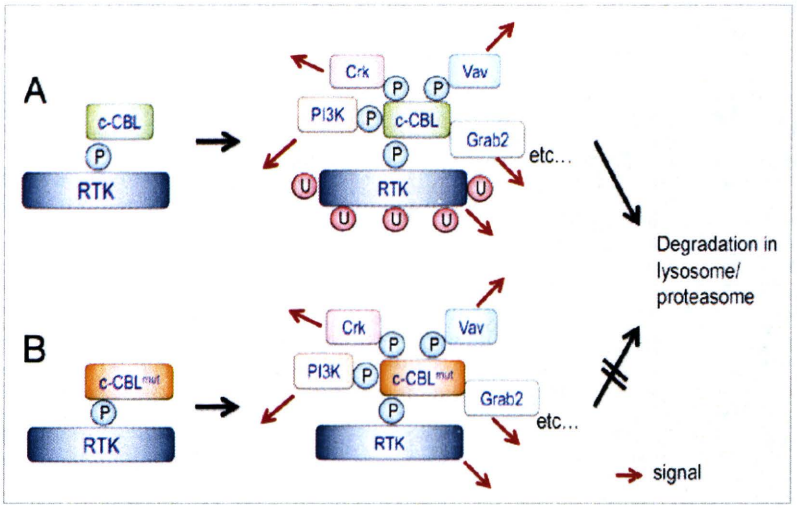

Figure 4. Positive regulation of signal transduction by c-CBL. (A) Having E3 ubiquitin ligase activity for negative regulation of signaling, c-CBL also works as an adaptor protein for multiple signal transduction molecules. When bound to phosphorylated tyrosine kinases, c-CBL is rapidly phosphorylated at multiple tyrosine residues, which in turn provide binding sites for a number of signal transduction molecules. Several lines of evidence suggest that binding to these molecules plays important roles in positive regulation of signal transduction (red arrows). Normally, phosphorylated c-CBL undergoes degradation, which is mediated by its E3 ubiquitin ligase activity. Thus, degradation of mutated c-CBL could be retarded, leading to prolonged transmission of positive signals (B).

References

1. Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2001; 2:294-307.
2. Schmidt MH, Dikic I. The Cbl interactome and its functions. Nat Rev Mol Cell Biol 2005; 6:907-18.
3. Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC, 3rd. v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci USA 1989; 86:1168-72.
4. Blake TJ, Shapiro M, Morse HC, 3rd, Langdon WY. The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene 1991; 6:653-7.
5. Abbas S, Rotmans G, Lowenberg B, Valk PJ. Exon 8 splice site mutations in the gene encoding the E3-ligase CBL are associated with core binding factor acute myeloid leukemias. Haematologica 2008; 93:1595-7.
6. Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 2008; 68:10349-57.
7. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 2009; 113:6182-92.
8. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood 2009; 114:1859-63.
9. Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature 2009; 460:904-8.
10. Van Etten RA, Shannon KM. Focus on myeloproliferative diseases and myelodysplastic syndromes. Cancer Cell 2004; 6:547-52.
11. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009; 114:937-51.
12. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005; 434:1144-8.
13. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005; 352:1779-90.
14. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005; 7:387-97.
15. Levine RL, Pardanani A, Tefferi A, Gilliland DG. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nat Rev Cancer 2007; 7:673-83.
16. Najfeld V, Montella L, Scalise A, Fruchtman S. Exploring polycythaemia vera with fluorescence in situ hybridization: additional cryptic 9p is the most frequent abnormality detected. Br J Haematol 2002; 119:558-66.
17. Kawamata N, Ogawa S, Yamamoto G, Lehmann S, Levine RL, Pikman Y, et al. Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray. Exp Hematol 2008; 36:1471-9.
18. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001; 1:157-62.

19. Caligiuri MA, Briesewitz R, Yu J, Wang L, Wei M, Arnoczky KJ, et al. Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. Blood 2007; 110:1022-4.
20. Sargin B, Choudhary C, Crosetto N, Schmidt MH, Grundler R, Rensinghoff M, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood 2007; 110:1004-12.
21. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 1999; 286:309-12.
22. Murphy MA, Schnall RG, Venter DJ, Barnett L, Bertoncello I, Thien CB, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. Mol Cell Biol 1998; 18:4872-82.
23. Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc Natl Acad Sci USA 1998; 95:15547-52.
24. Rathinam C, Thien CB, Langdon WY, Gu H, Flavell RA. The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells. Genes Dev 2008; 22:992-7.
25. Zheng N, Wang P, Jeffrey PD, Pavletich NP. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell 2000; 102:533-9.
26. Zeng S, Xu Z, Lipkowitz S, Longley JB. Regulation of stem cell factor receptor signaling by Cbl family proteins (Cbl-b/c-Cbl). Blood 2005; 105:226-32.
27. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat Immunol 2002; 3:1192-9.
28. Lang GA, Iwakuma T, Suh YA, Liu G, Rao VA, Parant JM, et al. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell 2004; 119:861-72.
29. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 2004; 119:847-60.
30. Marengere LE, Mirtsos C, Kozieradzki I, Veillette A, Mak TW, Penninger JM. Proto-oncoprotein Vav interacts with c-Cbl in activated thymocytes and peripheral T cells. J Immunol 1997; 159:70-6.
31. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J. Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia. J Biol Chem 1995; 270:21468-71.
32. Buday L, Khwaja A, Sipeki S, Farago A, Downward J. Interactions of Cbl with two adapter proteins, Grb2 and Crk, upon T cell activation. J Biol Chem 1996; 271:6159-63.
33. Sattler M, Salgia R, Okuda K, Uemura N, Durstin MA, Pisick E, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. Oncogene 1996; 12:839-46.
34. Ribon V, Hubbell S, Herrera R, Saltiel AR. The product of the cbl oncogene forms stable complexes in vivo with endogenous Crk in a tyrosine phosphorylation-dependent manner. Mol Cell Biol 1996; 16:45-52.
35. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, et al. SH2 domains recognize specific phosphopeptide sequences. Cell 1993; 72:767-78.
36. Hunter S, Burton EA, Wu SC, Anderson SM. Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. J Biol Chem 1999; 274:2097-106.
37. Hartley D, Corvera S. Formation of c-Cbl.phosphatidylinositol 3-kinase complexes on lymphocyte membranes by a p56lck-independent mechanism. J Biol Chem 1996; 271:21939-43.
38. Fukazawa T, Miyake S, Band V, Band H. Tyrosine phosphorylation of Cbl upon epidermal growth factor (EGF) stimulation and its association with EGF receptor and downstream signaling proteins. J Biol Chem 1996; 271:14554-9.
39. Jiang X, Huang F, Marusyk A, Sorkin A. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell 2003; 14:858-70.
40. Meisner H, Czech MP. Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. J Biol Chem 1995; 270:25332-5.
41. Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, et al. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 2000; 407:202-7.
42. Ueno H, Sasaki K, Honda H, Nakamoto T, Yamagata T, Miyagawa K, et al. c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3'-kinase pathway. Blood 1998; 91:46-53.
43. Grishin A, Sinha S, Roginskaya V, Boyer MJ, Gomez-Cambronero J, Zuo S, et al. Involvement of Shc and Cbl-PI 3-kinase in Lyn-dependent proliferative signaling pathways for G-CSF. Oncogene 2000; 19:97-105.
44. Garcia-Guzman M, Larsen E, Vuori K. The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. Oncogene 2000; 19:4058-65.
45. Tanaka S, Amling M, Neff L, Peyman A, Uhlmann E, Levy JB, et al. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 1996; 383:528-31.
46. Meng F, Lowell CA. A beta1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J 1998; 17:4391-403.
47. Scaife RM, Job D, Langdon WY. Rapid microtubule-dependent induction of neurite-like extensions in NIH 3T3 fibroblasts by inhibition of ROCK and Cbl. Mol Biol Cell 2003; 14:4605-17.
48. Gotoh T, Niino Y, Tokuda M, Hatase O, Nakamura S, Matsuda M, et al. Activation of R-Ras by Ras-guanine nucleotide-releasing factor. J Biol Chem 1997; 272:18602-7.
49. Bachmaier K, Krawczyk C, Kozieradzki I, Kong YY, Sasaki T, Oliveira-dos-Santos A, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature 2000; 403:211-6.
50. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. Nature 2000; 403:216-20.

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ORIGINAL ARTICLE

CD133 suppresses neuroblastoma cell differentiation via signal pathway modification

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CD133 (prominin-1) is a transmembrane glycoprotein expressed on the surface of normal and cancer stem cells (tumor-initiating cells), progenitor cells, rod photoreceptor cells and a variety of epithelial cells. Although CD133 is widely used as a marker of various somatic and putative cancer stem cells, its contribution to the fundamental properties of cancer cells, such as tumorigenesis and differentiation, remains to be elucidated. In the present report, we found that CD133 was expressed in several neuroblastoma (NB) cell lines/tumor samples. Intriguingly, CD133 repressed NB cell differentiation, for example neurite extension and the expression of differentiation marker proteins, and was decreased by several differentiation stimuli, but accelerated cell proliferation, anchorage-independent colony formation and *in vivo* tumor formation of NB cells. NB cell line and primary tumor-sphere experiments indicated that the molecular mechanism of CD133-related differentiation suppression in NB was in part dependent on neurotrophic receptor RET tyrosine kinase regulation. RET transcription was suppressed by CD133 in NB cells and glial cell line-derived neurotrophic factor treatment failed to induce RET in CD133-expressing cells; RET overexpression rescued CD133-related inhibition of neurite elongation. Of note, CD133-related NB cell differentiation and RET repression were mainly dependent on p38MAPK and PI3K/Akt pathways. Furthermore, CD133 has a function in growth and RET expression in NB cell line- and primary tumor cell-derived tumor spheres. To the best of our knowledge, this is the first report of the function of CD133 in cancer cells and our findings may be applied to improve differentiation induction therapy for NB patients.
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Introduction

CD133 (AC133; human prominin-1) belongs to a family of cell-surface glycoproteins harboring five transmembrane domains (Corbeil *et al.*, 2001) and was originally found as a hematopoietic stem cell marker (Yin *et al.*, 1997). CD133 was subsequently shown to be expressed by a number of progenitor cells, including those of the epithelium, where it is expressed on the apical surface (Corbeil *et al.*, 2000). Previously, it was found that CD133-expressing cells in brain tumors have the capacity for unlimited self-renewal, as well as the ability, in small numbers, to initiate tumor formation and progression in immuno-deficient mice (Singh *et al.*, 2004), suggesting that CD133-expressing cells satisfy the important criteria required for tumor-initiating cells (TICs) (Reya *et al.*, 2001; Jordan *et al.*, 2006). Using similar methods, CD133 has recently been designated as a marker associated with TICs in the colon (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), pancreatic (Olempska *et al.*, 2007), liver (Yin *et al.*, 2007), skin (Monzani *et al.*, 2007) and prostate (Collins *et al.*, 2005; Miki *et al.*, 2007) cancers. Maw *et al.* (2000) reported homozygosity for a 1-bp deletion (1878delG) in exon 16 of the CD133 gene predicted to cause a frameshift at codon 614 and a prematurely truncated protein lacking about half of the second extracellular loop, the final membrane-spanning segment and the cytoplasmic-C-terminal domain; this missense mutation caused retinal degeneration in four affected members of a consanguineous Indian family. This finding was further confirmed by an article describing that loss of Prom-1 in genetically modified mouse results in the progressive degeneration of mature photoreceptors with complete loss of vision (Zacchigna *et al.*, 2009); however, to the best of our knowledge, no reports have studied the function of CD133 in tumorigenesis.

Neuroblastoma (NB) is the most common pediatric solid malignant tumor derived from the sympathetic nervous system. Unlike the many childhood malignancies for which survival has been improved by recent therapies, high-risk NB is still one of the most difficult tumors to cure, with only 30% long-term survival despite intensive multimodal therapy (Maris *et al.*,

2007). The clinical presentation and treatment response of advanced NB, which results in relapse and a refractory state after a good responsive to the initial chemotherapy, suggest that TICs likely exist in NB tumors. A previous report indicated the isolation and characterization of putative TICs using primary-sphere formation with tumors and bone marrow metastases from NB patients, although CD133 expression was not detected in a bone marrow-derived high-risk NB tumor-sphere sample (Hansford *et al.*, 2007). On the other hand, it was reported that sub-cloned NB cells (designated 'intermediate type'), which have a significantly more malignant phenotype, with four- to fivefold greater plating efficiencies in soft agar and sixfold higher tumorigenicity in athymic mice, expressed high amounts of CD133 mRNA compared with less malignant subclones (Walton *et al.*, 2004); therefore, the function of CD133 in NB tumorigenesis and aggressiveness remains unresolved.

Previous reports about CD133 expression in NB and its function as a stem cell marker in several tumors prompted us to study the function of CD133 in NB cells (Walton *et al.*, 2004; Hansford *et al.*, 2007). Our results clearly indicated that CD133 also seems to regulate cell proliferation and tumorigenesis in NB cells. Importantly, CD133 represses NB cell differentiation and is decreased by several differentiation stimulators. We studied the molecular mechanism of CD133-related differentiation inhibition in NB cells and found that it was in part dependent on RET tyrosine kinase receptor regulation via signal pathway modification. Furthermore, CD133 is expressed in NB cell spheres and has a function in sphere growth and RET regulation.

In specific malignancies, for example NB and acute promyelocytic leukemia, differentiation induction therapy using retinoic acid is clearly effective. *In vitro* experiments indicated that all-*trans*-retinoic acid (ATRA) treatment induced morphological and biochemical differentiation in these cancer cells, suggesting that the induced differentiation seems to repress the tumorigenic activity of cancer cells (Brodeur *et al.*, 2000; Weinberg, 2006). Together, CD133 may regulate NB tumorigenesis and proliferation by preventing differentiation.

Results

CD133 has a function in NB cell proliferation

First, we checked the expression of CD133 in NB cell lines and found its expression in 7 out of 20 (53%) cell lines (Figure 3d and Supplementary Figure 1S). A high level of cell-surface expression of *CD133* was detected in TGW and SK-N-DZ cells, and modest expression was found in IMR32 (Figure 1a; Supplementary Figure 1Sa). Next, we knocked down *CD133* in highly expressing NB cells and analyzed the knockdown-induced phenotype. Figure 1b shows that infection of shRNA-reduced CD133 mRNA and protein and *CD133* knockdown in TGW cells effectively resulted in significant growth retardation. Inhibition of cell proliferation by CD133 small-interference RNA was also observed in SK-N-DZ cells (Supplementary Figure S1). Furthermore, stable knockdown of *CD133* in TGW cells suppressed cell proliferation under anchorage-independent conditions (Figure 1c). To test tumorigenicity *in vivo*, *CD133*-silenced TGW cells were injected subcutaneously into nude mice. Mock shRNA lentivirus-infected cells formed large tumors within 9 days post-injection; CD133 shRNA lentivirus-infected cells formed very small tumors (Figure 1d). Next, we examined the effect of CD133 on NB cell proliferation (Supplementary Figure 2S). CD133 was successfully expressed in SH-SY5Y cells by lentivirus. The proliferation rate of CD133-expressing SH-SY5Y cells was 2–2.5-fold greater than mock cells. Moreover, a soft agar colony formation assay showed that CD133-expressing cells formed more and bigger colonies than mock-control cells.

CD133 knockdown induces NB differentiation

In NB cells, differentiation into a neuronal phenotype is induced when cells are treated with several stimulations. Glial cell line-derived neurotrophic factor (GDNF) induced neurite outgrowth in TGW cells (Figure 2a, center). In CD133 knocked-down TGW cells, neurite formation was observed even under normal culture conditions (Figure 2a, KD). We scored cells with neurite length longer than the cell body diameter as neurite positive (Figure 2b). CD133 knocked-down cells showed intensified neurite extensions when compared with mock cells. Mock-infected and CD133 knocked-down cells were collected at the end of the experiment, and mRNA was extracted and subjected to RT–PCR (Figure 2c). With *GAP43/neurofilament (NF) 68* as neuronal differentiation markers, these expressions were constitutively upregulated in CD133 knocked-down cells. Along with differentiation induced by treatment with ATRA or phorbol-12-myristate-13-acetate (TPA) in parental TGW cells, CD133 expression was suppressed at both protein and mRNA levels (Supplementary Figure 3S). These results indicated that CD133 may suppress the differentiation of NB cells.

CD133 regulates RET expression in NB cells

To identify the mechanism of CD133-related cellular differentiation, we studied the expression of several neurotrophic receptors and RET receptors because they are the important signal transduction pathway molecules, which have important functions in sympathetic nerve and NB cell differentiation (Kaplan *et al.*, 1993; Klein, 1994; D'Alessio *et al.*, 1995; Enomoto *et al.*, 2001). We introduced *CD133* cDNA into several NB cell lines (Figure 3a), and checked the effect of CD133 overexpression on *RET* expression using a primer pair recognizing all *RET* isoforms, *RET51*, *RET9* and *RET43*, formed by alternative splicing of C-terminal exon cassettes (Myers *et al.*, 1995; Enomoto *et al.*, 2000). Intriguingly, in *RET* and all *RET* isoforms, transcriptions were suppressed in CD133-overexpressing NB cells (*RET* reduction was 1.3–3.8-fold by qPCR); however,

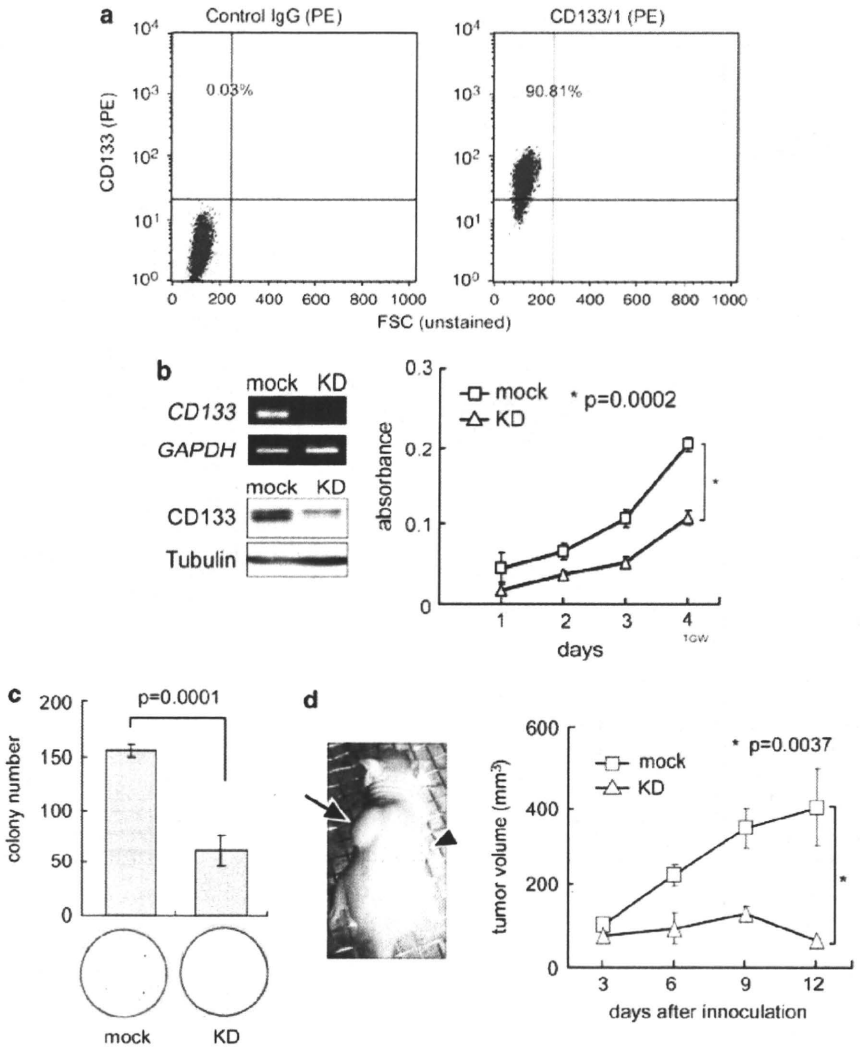

Figure 1 *CD133* knockdown inhibits the growth of human neuroblastoma (NB) cells. (**a**) Flow cytometric analysis of CD133 expression profiles in TGW cells. CD133 fluorescence is depicted on the *y* axis, and the percentage of CD133-positive cells is shown in the left upper corner of each plot. (**b**) Stable knockdown of *CD133* by lentivirus-mediated shRNA was performed as described in Materials and methods. *CD133* expression was detected by semi-quantitative RT–PCR and western blotting analysis in TGW cells. Growth curves were obtained by WST-8 assay. Anchorage-independent colony formation (**c**) and *in vivo* tumorigenic assay (**d**). TGW cells were stably transduced with shRNA against mock or CD133 (KD). (**c**) Colonies were stained with MTT dye and directly counted under a phase contrast microscope. (**d**) Tumor development in BALB/c AJcl *nu/nu* mice on injection of TGW cells stably infected with shRNA against mock (arrow) and CD133 (KD, arrowhead) cells. Tumor volume was measured every 3 days. Data are presented as the mean ± s.d. of tumors in four mice.

the effects of CD133 on *TrkA/B/C, p75NGFR* and *GDNF* expressions did not show a specific tendency. CD133 knockdown clearly increased *RET* mRNA (*RET* induction was 2.5–3.0-fold by qPCR). CD133-mediated RET downregulation was also observed at the protein level (Figure 3b). Furthermore, CD133 expression in primary NB spheres resulted in transcriptional suppression of *RET* (Figure 3c). These results suggest that CD133 suppresses *RET* gene transcription in NB cells.

To study the expression pattern of *CD133* and *RET* mRNA in human NBs, we performed semi-quantitative RT–PCR. *CD133* was expressed in 7 of 20 NB cell lines tested (Figure 3d), and only 1 NB cell line was *RET* positive in the 7 cell lines. We further studied *CD133* and *RET* expression in unfavorable patient-derived tumors (stages 3 and 4, TrkA(−), *MYCN* amplified). Again, *RET* expression was profoundly repressed in CD133-expressing NB tumors (Figure 3e). Finally, we studied the transcriptional activity of *RET* promoter in CD133-expressing cells. *RET* promoter reporter-derived luciferase activity was significantly suppressed in CD133-expressing cells (Figure 3e).

CD133 regulates NB cell differentiation in a RET-dependent manner

We investigated the biological effects of CD133 overexpression on *RET* downregulation in SH-SY5Y cells.

Significant neurite outgrowth was observed when mock-infected cells were stimulated with GDNF (Figure 4a). At the same time, no obvious difference was observed between mock- and GDNF-treated CD133-expressing cells. These results implied that CD133 overexpression inhibited NB cell differentiation.

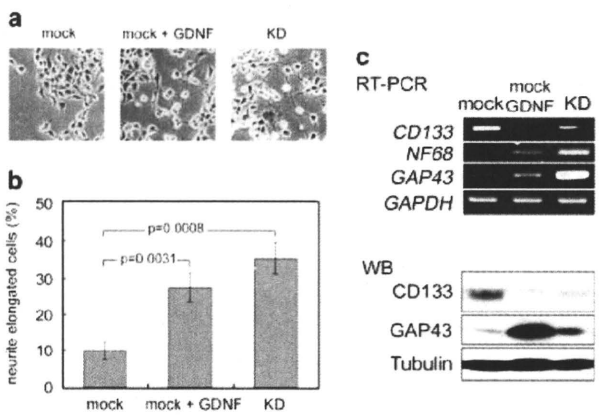

Figure 2 CD133 silencing induces differentiation in TGW cells. TGW cells were infected with lentivirus vectors encoding shRNA against *CD133* (right) or a mock (left) as a negative control. Ten days after infection, cells were treated with buffer (mock and KD) or GDNF (10 ng/ml, middle). Cells were scored for the presence of neurites longer than one cell diameter 72 h after treatment (photo: (**a**), bar graphs: (**b**)). Data are presented as the mean ± s.d. from at least three independent experiments. Statistical analysis was performed by Student's *t*-test. (**c**) NB differentiation-related molecule *neurofilament 68 (NF68)* and *GAP43* expressions in RT–PCR and WB. NF68 protein was not detected by WB in TGW cells.

We examined the effect of the co-expression of CD133 and RET (RET9) on SH-SY5Y cells. RET-expressing lentivirus was co-infected into stably CD133-expressing SH-SY5Y cells. Ten days after infection, ectopic RET and CD133 expressions were observed both at protein

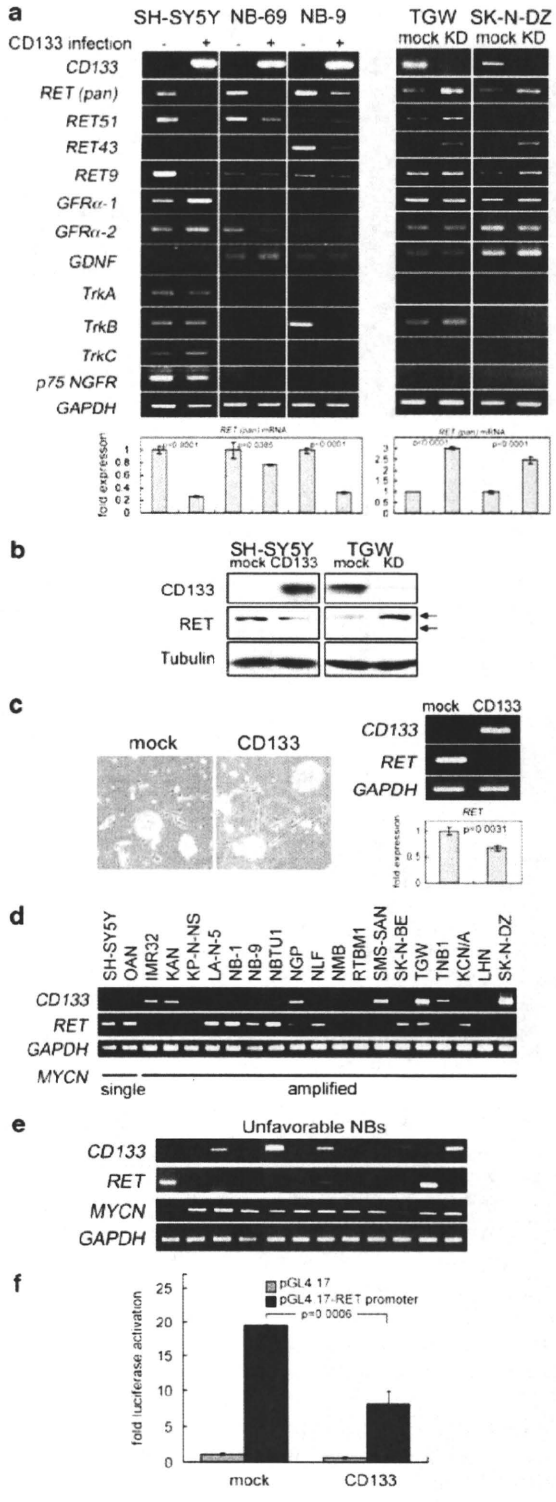

Figure 3 CD133 inhibits RET expression in NB cells. (**a**) SH-SY5Y, NB-69 and NB-9 cells were infected with mock or CD133-expressing lentivirus, and TGW and SK-N-DZ cells were stably infected with shRNA against mock or CD133 (KD) lentivirus. Semi-quantitative RT–PCR analyses were performed with CD133-modified NBs using specific primers against each *RET* isoform, *Trk* families, *GFRα-1/2* and *GDNF*. *GAPDH* was used as a loading control. Expression level of *RET* (pan) was analyzed by qPCR. In qPCR, relative *RET* values were normalized by *GAPDH*. Data are representative results of at least three independent experiments. (**b**) CD133-expressing SH-SY5Y or CD133 knocked-down TGW cell lysates were subjected to western blotting for CD133 and pan-RET expression. Pan-RET antibody detected two bands corresponding to RET isoforms (arrows). (**c**) Primary sphere from a stage 4 NB patient was infected with mock or CD133-expressing lentivirus. Five days after infection, RNA was extracted for semi-quantitative RT–PCR of *CD133*/*RET* and qPCR of *RET*. GAPDH was used as an internal control. Data are representative of three tumor samples. (**d**) Expression of *CD133* and *RET* mRNA in NB cell lines. In all, 18 NB cell lines with amplified *MYCN* and 2 cell lines with a single copy of *MYCN* were used for semi-quantitative RT–PCR analysis. (**e**) Semi-quantitative RT–PCR analysis in unfavorable primary NBs. The results of 12 NBs are shown. Unfavorable NBs: International NB Staging System (INSS) stage 3 or 4, *TrkA* (−), with *MYCN* amplified. (**f**) Effects of CD133 on *RET* promoter (0.8 kb) activity in SH-SY5Y cells. pGL4.17-*RET* promoter-driven luciferase activities were normalized to pRL-SV40 early enhancer/promoter-driven *Renilla* luciferase activities as the transfection control and expressed as relative values.

and mRNA levels (Figure 4b and data not shown). As seen in Figure 4a, GDNF significantly induced neurite outgrowth of CD133/RET co-expressing SH-SY5Y cells. CD133 single-infected cells did not respond to GDNF, suggesting that the response was dependent on RET receptor expression. However, the expression of neuronal cell differentiation markers induced by GDNF was not recovered by *RET* in CD133-expressing cells (Figure 4b). These findings indicated that CD133 inhibits GDNF-promoted neuronal differentiation via not only by *RET* but also by the other signal pathways.

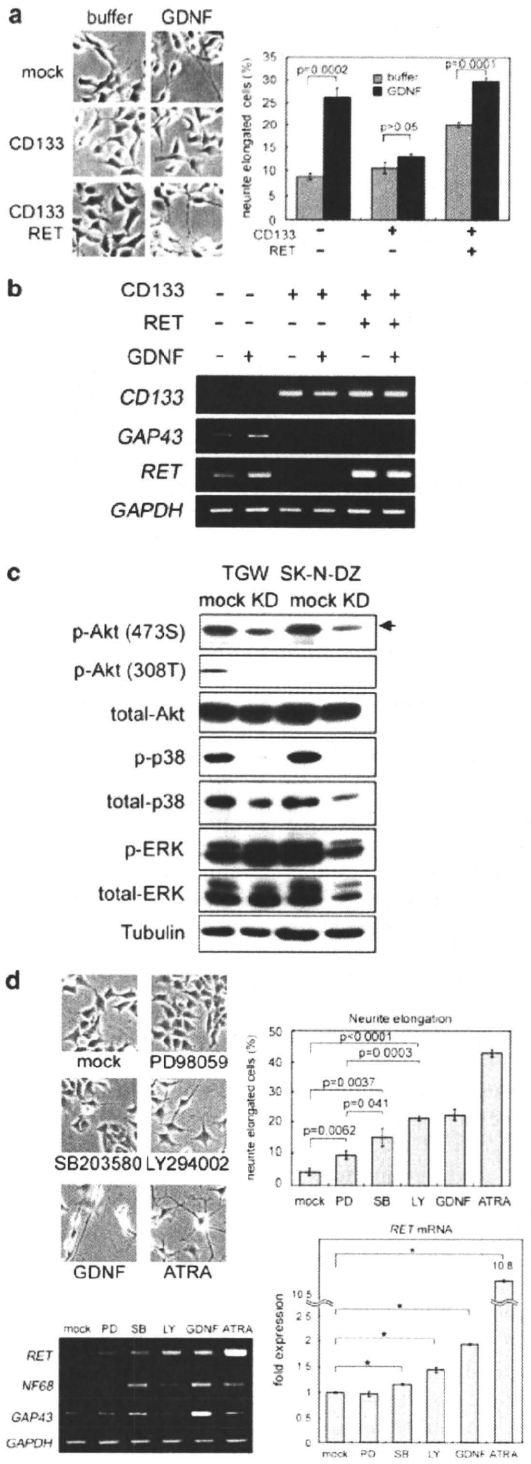

Figure 4 NB cell differentiation was regulated by CD133-dependent RET suppression via signal pathway modification. (**a**) Mock, CD133 and/or RET9 co-expressing SH-SY5Y cells were treated with GDNF (50 ng/ml) for 72 h. Cells were scored for the presence of neurites longer than one cell diameter after GDNF treatment. (**b**) CD133 and/or RET9 co-infected SH-SY5Y cells were cultured with or without GDNF treatment. Semi-quantitative RT–PCR analyses of *CD133*, *GAP43*, *RET* and *GAPDH* were performed. (**c**) The levels of phospho-Akt (p-Akt(473S) and p-Akt(308 T)), total-Akt, phospho-p38MAPK (p-p38), total-p38MAPK, phospho-ERK (p-ERK), total-ERK and tubulin were analyzed by western blot analysis. (**d**) TGW cells were cultured with DMSO (mock, 0.1%), PD98059 (PD, 5 μM), SB203580 (SB, 5 μM), LY294002 (LY, 5 μM), GDNF (50 ng/ml) or ATRA (5 μM) for 96 h. Cells were scored for the presence of neurite longer than one cell diameter after treatments. Semi-quantitative RT–PCR analysis of *RET*/*NF68*/*GAP43*/*GAPDH*, and qPCR of *RET* were performed.

CD133 regulates RET expression and NB cell differentiation by modification of signaling pathways

To identify the mechanism of *RET* downregulation in CD133-expressing cells, we studied the signaling molecule status in CD133 knocked-down cells (Figure 4c) and found a strong suppression of Akt (473S, 308T) and p38MAPK phosphorylation, but not ERK1/2 in both TGW and SK-N-DZ cells. To confirm the Akt and p38MAPK phosphorylation status caused by CD133 downregulation, we treated TGW cells with kinase inhibitors. MEK1 inhibitor (PD98059, PD), p38MAPK inhibitor (SB203580, SB) and PI3K inhibitor (LY294002, LY) induced neurite elongation in NB cells, and SB and LY were more effective for neurite elongation than PD. *RET* induction by kinase inhibitors was correlated with neurite elongation; however, differentiation markers *NF68* and *GAP43* were significantly induced by SB treatment. These results suggest that downregulation of p38MAPK and PI3K/Akt pathways has a function in CD133-related neurite elongation and differentiation marker expression is affected mainly by the p38MAPK pathway.

CD133 has a function in tumor-sphere growth and cell survival

It was previously reported that NB TICs were accumulated in NB spheres in serum-free media (SFM) (Hansford *et al.*, 2007). These observations prompted us to study the function of CD133 in tumor-sphere formation of NB cells. In IMR32 cells, only a small fraction of cells expressed CD133 (Supplementary Figure 1Sa). IMR32 cells were cultured in SFM with epidermal growth factor and fibroblast growth factor for a week, and sphere formation, upregulation of *CD133* (11.8-fold induction) and suppression of *RET* (2.8-fold reduction) were observed (Figure 5a). In primary NB cells from bone marrow metastasis,

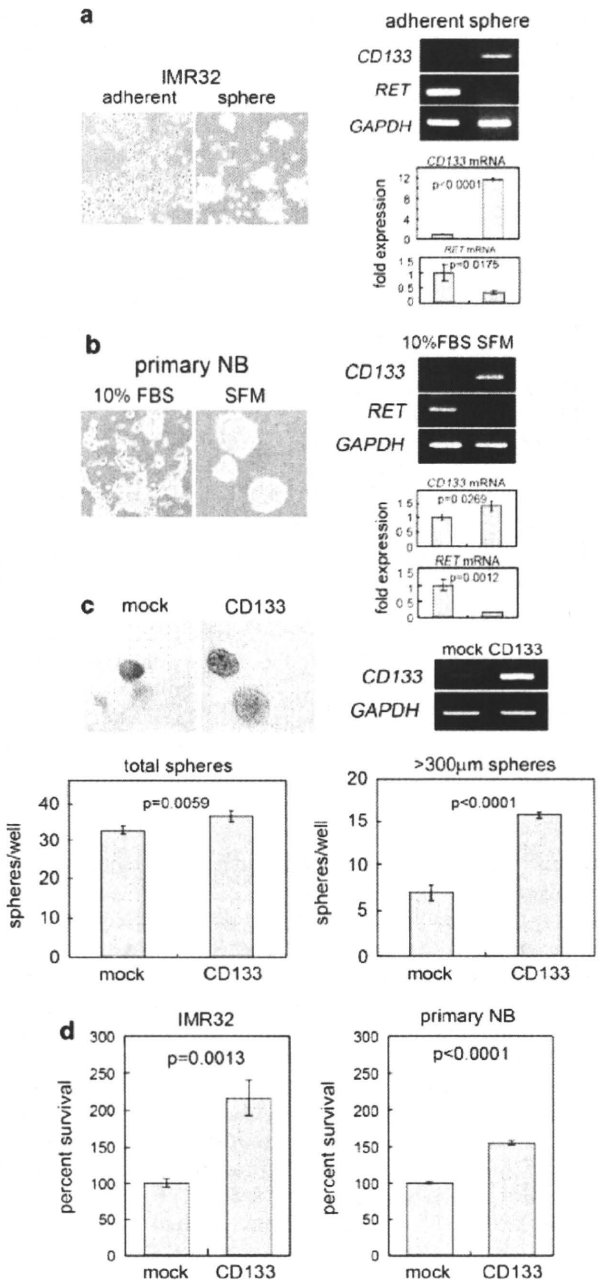

Figure 5 CD133 enhances cellular survival of NB cells in neurospheres. IMR32 cells (**a**) and primary NB cells (**b**) were cultured in 10% fetal bovine serum containing medium (adherent) or SFM (sphere) for a week. Semi-quantitative RT–PCR and qPCR analyses were performed with adherent or sphere cell RNAs using specific primers for *CD133* and *RET*. *GAPDH* was used as a loading control. Primary NB cell results are representative of three tumor samples. (**c**) IMR32 cells were stably infected with mock or CD133-expressing lentivirus. The expression levels of *CD133* and *GAPDH* were determined by semi-quantitative RT–PCR. Cells were cultured in 96-well culture plates with SFM. After 5 weeks, spheres were measured and counted under a microscope with an eyepiece micrometer. (**d**) Enzymatically dissociated IMR32- or primary NB-sphere cells were stained with trypan blue and counted to determine the number of viable cells.

the upregulation of CD133 (1.4-fold induction) and downregulation of RET receptors (5.9-fold reduction) were found in SFM medium, but not in the medium with 10% fetal bovine serum (Figure 5b). Next, we introduced CD133 into IMR32 cells by lentivirus infection, and after 5-week culture in SFM, we found that CD133-expressing cells formed many more large spheres than mock cells (Figure 5c). Moreover, CD133-expressing spheres made from IMR32 and primary NB cells contained many more living cells than the mock control (Figure 5d), suggesting that CD133 promotes NB cell survival in tumor-sphere formation.

Discussion

Increasing evidence highlights the function of CD133 as a marker of CSCs in various human tumors; however, its function in tumorigenesis remains to be elucidated by molecular biology experiments. In this study, CD133-knockdown experiments indicated that CD133 represses differentiation in NB cells; CD133 was clearly decreased by differentiation-inducing stimulation, for example ATRA and TPA treatments. Brodeur *et al.* (2000) indicated that neurotrophic factors and their receptors have a significant function in NB behavior and the potential to send intracellular signals into the nucleus to produce neuronal differentiation in the normal sympathetic nerve system. Among the NB cell differentiation-related neurotrophic receptors, *RET* transcription was regulated by CD133 in NB cell lines. The expression of CD133 effectively inhibited NB cell differentiation (neurite extension and differentiation markers). Furthermore, RET expression partly rescued the CD133-related inhibition of differentiation. These findings suggest that CD133-mediated RET suppression has a considerable function in NB cell differentiation. Regarding the function of RET in NB differentiation, Peterson and Bogenmann (2004) suggested that RET receptor activation inhibits cell cycle progression and enhances responsiveness to NGF; thus, NB cell differentiation requires the collaboration of functional RET and TrkA signal pathways; they also reported that GDNF treatment induced *RET* transcription in NB cells. Intriguingly, our results indicate that CD133 expression effectively suppressed *RET* mRNA in NB cells and CD133 knockdown induced NB cell differentiation, suggesting that suppression of CD133 by small-interference RNA administration will increase *RET* transcription in CD133-expressing NB tumors and may be useful in differentiation induction therapy for resistant- and relapsed-NB tumors. In addition, transcriptional suppression of *CD133* could be useful to induce differentiation in NB cells; however, the exact mechanism of transcriptional regulation of CD133 has not been clarified. Although seven CD133 mRNA isoforms controlled by five alternative promoters were reported previously (Shmelkov *et al.*, 2004), the promoter activities of these isoforms were studied only by pGL3-enhancer vector, suggesting the existence of other *cis*-elements in the CD133 locus.

Several studies have been reported to elucidate the molecular mechanism and signaling pathways that regulate the behavior of CD133-expressing cancer cells. Nikolova *et al.* (2007) reported that WNT-conditional media had effects on the proliferation and differentiation of cord blood-derived CD133-positive cells, and Fan *et al.* (2006) showed that Notch signal inhibition by GSI-18 reduced the CD133-positive fraction in brain tumor cells. Regarding the analysis of the intracellular signaling pathway related to the CD133 function, one report suggested the significance of the Akt/PKB pathway in the expression of survival proteins, phosphor-Bad and Bcl-2 in CD133-positive hepatocellular carcinoma cell survival (Ma *et al.*, 2007). In our study, CD133-knockdown experiments indicated that CD133-related RET repression and NB cell differentiation were caused by signal pathway activation, for example p38MAPK and PI3K/Akt pathways. To support this observation, treatment with kinase inhibitors showed a correlation between neurite elongation and RET induction in NB cells, and that differentiation marker protein induction was mainly dependent on the p38MAPK pathway. These findings suggest that CD133 prevents NB cell differentiation via signal transduction pathways. To the best of our knowledge, this is the first report of CD133-related signal pathway modification resulting in cell differentiation. As CD133 is a membranous protein on stem cells and cancer stem cells, it is possible that CD133 affects membranous receptor functions and the downstream signal pathways. In addition, Boivin *et al.* (2009) reported the phosphorylation of CD133-cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. Site-directed mutagenesis of these tyrosine residues in CD133 will provide important information for CD133 functions in our experimental system using NB cells.

Materials and methods

Cell culture and reagents

Human NB cell lines were obtained from official cell banks (RIKEN Cell Bank, Tsukuba, Japan and ATCC, Manassas, VA, USA) and cultured in high-glucose DMEM (Sigma-Aldrich, St Louis, MO, USA) or RPMI1640 (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 50 μg/ml penicillin/streptomycin (Sigma-Aldrich) in an incubator with humidified air at 37 °C with 5% CO_2. NB cell lines subjected to molecular biology and biochemistry experiments were MYCN single-copy SH-SY5Y cells and MYCN-amplified TGW, SK-N-DZ and IMR32 cells. GDNF was obtained from Invitrogen. ATRA was from Sigma-Aldrich. Phorbol-12-myristate-13-acetate (TPA) was from Nacalai Tesque (Kyoto, Japan). LY294002 was from Cell Signaling Technology (Beverly, MA, USA). PD98059 and SB203580 were from Calbiochem (San Diego, CA, USA).

Fluorescence-activated cell sorting analysis

NB cell lines growing in the log phase were enzymatically removed from 10 cm diameter culture dishes, washed with cold PBS and treated with biotinylated AC133 (CD133/1) monoclonal antibodies (Miltenyi Biotec, Auburn, CA, USA) or control IgG2A (eBioscience, San Diego, CA, USA) for 15 min at 4 °C. The primary antibody was removed, and then the cells were washed twice with ice-cold PBS containing 0.1% BSA, and a 1:200 dilution of phycoerythrin-labeled streptavidin (eBioscience) added for 15 min at 4 °C. After washing, flow cytometry was performed using a fluorescence-activated cell sorting Caliber (BD, San Jose, CA, USA).

Knockdown of CD133

For RNAi experiments, predesigned, double-stranded SMART-pool small-interference RNA targeting human *CD133* (*prominin-1*) was purchased from Dharmacon (Lafayette, CO, USA) and Silencer Negative Control small-interference RNA #1 was purchased from Ambion (Austin, TX, USA).

Lentivirus-mediated gene transduction and knockdown

The packaging cell line HEK 293T (4×10^6) was plated and transfected the following day. Then, 1.5 μg transducing vectors containing the gene [pHR-SIN-CMV-G-DL1 or CSII-CMV-MCS-IRES2-Bsd vector (RIKEN Bioresource Center, Ibaraki, Japan)] or shRNA [pLKO.1 (Sigma-Aldrich)] and 2.0 μg packaging vectors (Sigma-Aldrich) were co-transfected with Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocols. The medium was changed the following day, and cells were cultured for another 24 h. Conditioned medium was collected and cleared of debris by filtering through a 0.45 μm filter (Millipore, Bedford, MA, USA). Then, 1×10^5 NB cells were seeded in each well of a six-well plate, and transduced by lentiviral-conditioned media. Transduced cells were analyzed by western blotting and RT–PCR.

Cloning of human CD133 cDNA

The human *CD133* cDNA (RefSeq NM_006017) was cloned from human colon cancer cell line Caco-2 mRNA by RT–PCR using specific primer sets described in Supplementary Table 1S. *CD133* cDNA fragment was sub-cloned into a lentiviral-based vector (pHR-SIN-CSGW) (Hasegawa *et al.*, 2006).

Western blot analysis

The cells were lysed in buffer containing 5 mM EDTA, 2 mM Tris–HCl (pH 7.5), 10 mM β-glycerophosphate, 5 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, a protease inhibitor cocktail (Nacalai Tesque) and 1% SDS. Western blot analysis was performed as reported previously (Kurata *et al.*, 2008). For CD133 detection, we used AC133 monoclonal antibody. Anti-RET (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho- and total Akt, p38, ERK (Cell Signaling Technology) and anti-tubulin antibody from Lab Vision (Fremont, CA, USA) were also used.

Semi-quantitative RT–PCR

Semi-quantitative RT–PCR analysis was as described previously (Kurata *et al.*, 2008). Total cellular RNA for preparing RT–PCR templates was extracted using ISOGEN (Nippon Gene KK, Tokyo, Japan). The cDNA was synthesized from 1 μg total RNA and then subjected to PCR. Primer sequences are described in Supplementary Table 1S. RT–PCR results are representative of at least three independent experiments.

qPCR analysis

The qPCR analysis was performed as described previously (Ochiai *et al.*, 2010). The primers for qPCR were designed and synthesized to produce 50–150 bp products. The primer sequence is listed in Supplementary Table 1S. The results were representative of at least three independent experiments.

Cell proliferation and soft agar assay
Cells were seeded into 96-well plates (750 per well) in culture medium containing 10% fetal bovine serum. Every 24 h, cell viability was determined by water-soluble tetrazolium salt (WST-8) assay using Counting kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. For soft agar assay, 2×10^3 cells of stable infectants TGW or SH-SY5Y cells were seeded in soft agar as described previously (Aoyama *et al.*, 2005). Viable colonies were stained with 0.05 mg/ml MTT.

Tumor formation in nude mice
For tumor formation, 6-week-old female athymic BALB/c AJcl *nu/nu* mice (CLEA Japan, Shizuoka, Japan) were injected into the femur with 1×10^7 TGW cells as described previously (Aoyama *et al.*, 2005). The handling of animals was in accordance with the guidelines of Chiba Cancer Center Research Institute.

Patients and tumor specimens
The 12 tumor specimens used in this study were kindly provided by various institutions and hospitals in Japan. Informed consent was obtained at each institution or hospital. All tumors were diagnosed clinically as well as pathologically as NB and staged according to the International NB Staging System criteria. The patients were treated by standard chemotherapy protocols as described previously (Kaneko *et al.*, 2002; Iehara *et al.*, 2006). *MYCN* copy number, *TrkA* mRNA expression levels and DNA index were measured as reported previously (Ohira *et al.*, 2003). This study was approved by the Institutional Review Board of Chiba Cancer Center.

Subcloning of human RET (RET9)
Human *RET9* (Crowder *et al.*, 2004) full-length cDNA was a kind gift from Dr Hideki Enomoto (RIKEN Center for Developmental Biology, Hyogo, Japan). *RET9* cDNA fragment (3.4 kb) was sub-cloned into the *Not*I site of CSII-CMV-MCS-IRES2-Bsd vector, which had been altered to accept the *Xba*I and *Hin*dIII ends.

Cloning of human RET promoter
Human *RET* promoter 1.5 kb (−919 to +550, position +1 is the transcription start site determined in a previous report (Itoh *et al.*, 1992)) was amplified from human genomic DNA using Platinum *Pfx* polymerase (Invitrogen) with primers (described in Supplementary Table 1S) by PCR amplification and sub-cloned into pGEM-T easy vector (Promega, Southampton, UK). The *RET* 5′-flanking sequence from −453 to +227 was sub-cloned into the *Eco*RV site of pGL4.17 reporter vector (Promega).

Sphere culture of NB cells
The preparation of primary NB cells from stage 4 patients' bone marrow was described previously (Nakanishi *et al.*, 2007). Dissociated primary NB cells or IMR32 cells were cultured in SFM (DMEM-F12, 1:1 (Wako), 50 μg/ml penicillin/streptomycin, 2% B27 supplement (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml fibroblast growth factor basic (Invitrogen)). Half of the medium was replaced with fresh culture medium every 7 days. IMR32 cells and primary NB cells were seeded in 96-well (400 per well) or six-well (1×10^5 per well) and six-well (1.7×10^5 per well) plates, respectively. Spheres were counted and measured under a microscope with an eyepiece micrometer. Five-week cultured IMR32 or 2-month cultured primary NB spheres were dissociated by Accumax (Innovative Cell Technologies, San Diego, CA, USA) according to the manufacturer's protocol. Living cells were counted based on morphological criteria and trypan blue staining.

Conflict of interest

The authors declare no conflict of interest.

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References

Aoyama M, Ozaki T, Inuzuka H, Tomotsune D, Hirato J, Okamoto Y *et al.* (2005). LMO3 interacts with neuronal transcription factor, HEN2, and acts as an oncogene in neuroblastoma. *Cancer Res* **65**: 4587–4597.

Boivin D, Labbé D, Fontaine N, Lamy S, Beaulieu E, Gingras D *et al.* (2009). The stem cell marker CD133 (prominin-1) is phosphorylated on cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. *Biochemistry* **48**: 3998–4007.

Brodeur GM, Sawada T, Tsuchida Y, Voute PA (eds) (2000). *Neuroblastoma*. Elsevier Science: Amsterdam.

Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* **65**: 10946–10951.

Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM *et al.* (2000). The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem* **275**: 5512–5520.

Corbeil D, Fargeas CA, Huttner WB. (2001). Rat prominin, like its mouse and human orthologues, is a pentaspan membrane glycoprotein. *Biochem Biophys Res Commun* **285**: 939–944.

Crowder RJ, Enomoto H, Yang M, Johnson Jr EM, Milbrandt J. (2004). Dok-6, a Novel p62 Dok family member, promotes Ret-mediated neurite outgrowth. *J Biol Chem* **279**: 42072–42081.

D'Alessio A, De Vita G, Cali G, Nitsch L, Fusco A, Vecchio G *et al.* (1995). Expression of the RET oncogene induces differentiation of SK-N-BE neuroblastoma cells. *Cell Growth Differ* **6**: 1387–1394.

Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson Jr EM, Milbrandt J. (2001). RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. *Development* **128**: 3963–3974.

Enomoto H, Heuckeroth RO, Golden JP, Johnson EM, Milbrandt J. (2000). Development of cranial parasympathetic ganglia

requires sequential actions of GDNF and neurturin. *Development* **127**: 4877–4889.

Fan X, Matsui W, Khaki L, Stearns D, Chun J, Li YM *et al.* (2006). Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer Res* **66**: 7445–7452.

Hansford LM, McKee AE, Zhang L, George RE, Gerstle JT, Thorner PS *et al.* (2007). Neuroblastoma cells isolated from bone marrow metastases contain a naturally enriched tumor-initiating cell. *Cancer Res* **67**: 11234–11243.

Hasegawa K, Nakamura T, Harvey M, Ikeda Y, Oberg A, Figini M *et al.* (2006). The use of a tropism-modified measles virus in folate receptor-targeted virotherapy of ovarian cancer. *Clin Cancer Res* **12**: 6170–6178.

Itoh F, Ishizaka Y, Tahira T, Yamamoto M, Miya A, Imai K *et al.* (1992). Identification and analysis of the *ret* proto-oncogene promoter region in neuroblastoma cell lines and medullary thyroid carcinomas from MEN2A patients. *Oncogene* **7**: 1201–1206.

Iehara T, Hosoi H, Akazawa K, Matsumoto Y, Yamamoto K, Suita S *et al.* (2006). MYCN gene amplification is a powerful prognostic factor even in infantile neuroblastoma detected by mass screening. *Br J Cancer* **94**: 1510–1515.

Jordan CT, Guzman ML, Noble M. (2006). Cancer stem cells. *N Engl J Med* **355**: 1253–1261.

Kaplan D, Matsumoto K, Lucarelli E, Thiele CJ. (1993). Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group. *Neuron* **11**: 321–331.

Kaneko M, Tsuchida Y, Mugishima H, Ohnuma N, Yamamoto K, Kawa K *et al.* (2002). Intensified chemotherapy increases the survival rates in patients with stage 4 neuroblastoma with MYCN amplification. *J Pediatr Hematol Oncol* **24**: 613–621.

Klein R. (1994). Role of neurotrophins in mouse neuronal development. *FASEB J* **8**: 738–744.

Kurata K, Yanagisawa R, Ohira M, Kitagawa M, Nakagawara A, Kamijo T. (2008). Stress via p53 pathway causes apoptosis by mitochondrial Noxa upregulation in doxorubicin-treated neuroblastoma cells. *Oncogene* **27**: 741–754.

Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. (2007). CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. *Oncogene* **27**: 1749–1758.

Maris JM, Hogarty MD, Bagatell R, Cohn SL. (2007). Neuroblastoma. *Lancet* **369**: 2106–2120.

Maw MA, Corbeil D, Koch J, Hellwig A, Wilson-Wheeler JC, Bridges RJ *et al.* (2000). A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. *Hum Mol Genet* **9**: 27–34.

Miki J, Furusato B, Li H, Gu Y, Takahashi H, Egawa S *et al.* (2007). Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor derived human prostate epithelial cell lines and in prostate cancer specimens. *Cancer Res* **67**: 3153–3161.

Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C *et al.* (2007). Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. *Eur J Cancer* **43**: 935–946.

Myers SM, Eng C, Ponder BA, Mulligan LM. (1995). Characterization of RET proto-oncogene 3′ splicing variants and polyadenylation sites: a novel C-terminus for RET. *Oncogene* **11**: 2039–2045.

Nakanishi H, Ozaki T, Nakamura Y, Hashizume K, Iwanaka T, Nakagawara A. (2007). Purification of human primary neuroblastomas by magnetic beads and their *in vitro* culture. *Oncol Rep* **17**: 1315–1320.

Nikolova T, Wu M, Brumbarov K, Alt R, Opitz H, Boheler KR *et al.* (2007). WNT-conditioned media differentially affect the proliferation and differentiation of cord blood-derived CD133+ cells *in vitro*. *Differentiation* **75**: 100–111.

O'Brien CA, Pollett A, Gallinger S, Dick JE. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**: 106–110.

Ochiai H, Takenobu H, Nakagawa A, Yamaguchi Y, Kimura M, Ohira M *et al.* (2010). Bmi1 is a MYCN target gene that regulates tumorigenesis through repression of *KIF1Bβ* and *TSLC1* in neuroblastoma. *Oncogene* **29**: 2681–2690.

Ohira M, Morohashi A, Inuzuka H, Shishikura T, Kawamoto T, Kageyama H *et al.* (2003). Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets. *Oncogene* **22**: 5525–5536.

Olempska M, Eisenach PA, Ammerpohl O, Ungefroren H, Fandrich F, Kalthoff H. (2007). Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary Pancreat Dis Int* **6**: 92–97.

Peterson S, Bogenmann E. (2004). The RET and TRKA pathways collaborate to regulate neuroblastoma differentiation. *Oncogene* **23**: 213–225.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–111.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C *et al.* (2007). Identification and expansion of human colon-cancer initiating cells. *Nature* **445**: 111–115.

Shmelkov SV, Jun L, St Clair R, McGarrigle D, Derderian CA, Usenko JK *et al.* (2004). Alternative promoters regulate transcription of the gene that encodes stem cell surface protein AC133. *Blood* **103**: 2055–2061.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T *et al.* (2004). Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Walton JD, Kattan DR, Thomas SK, Spengler BA, Guo HF, Biedler JL *et al.* (2004). Characteristics of stem cells from human neuroblastoma cell lines and in tumors. *Neoplasia* **6**: 838–845.

Weinberg RA (ed) (2006). *The Biology of Cancer*. Garland Science: New York.

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG *et al.* (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90**: 5002–5012.

Yin S, Li J, Hu C, Chen X, Yao M, Yan M *et al.* (2007). CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int J Cancer* **120**: 1444–1450.

Zacchigna S, Oh H, Wilsch-Bräuninger M, Missol-Kolka E, Jászai J, Jansen S *et al.* (2009). Loss of the cholesterol-binding protein prominin-1/CD133 causes disk dysmorphogenesis and photoreceptor degeneration. *J Neurosci* **29**: 2297–2308.

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Concordance for neuroblastoma in monozygotic twins: case report and review of the literature

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Neuroblastoma;
Monozygotic twin;
Twin-to-twin metastasis

Abstract The patients were infant male twins born by cesarean delivery following a healthy pregnancy at 36 weeks' gestation to unrelated parents. At 4 months of age, twin 2 presented with hepatomegaly and a right suprarenal mass. Resection of an adrenal tumor and a liver tumor biopsy were performed. Twin 1 had no symptoms at 4 months of age. Screening by abdominal ultrasonography showed multiple masses in the liver but no adrenal mass. Metaiodobenzylguanidine scintigraphy showed positive findings in multiple liver masses. A laparoscopic biopsy for a liver tumor was performed. All primary tumor and liver tumor specimens from twin 2 and the liver tumor of twin 1 had the same histologic classification of neuroblastoma and nearly identical genetic aberrations, including a chromosome gain or loss using array–comparative genomic hybridization. From these clinical and pathologic findings and genetic analyses, we strongly demonstrate the transplacental metastatic spread from twin 2 to twin 1. In the literature, 9 pairs of concordant twin neuroblastomas, including the current twin, have been presented; and the clinical findings of 5 twin pairs may represent placental metastases from one twin with congenital neuroblastoma to the other twin. This study is the first report presenting the possibility of twin-to-twin metastasis in monozygotic twins with neuroblastoma based on an analysis of the clinical features and genetic aberrations.

Neuroblastoma is the most common neonatal solid abdominal tumor [1]. The tumor originates from the neural crest cells of the adrenal medulla or sympathetic ganglia. The neuroblastoma concordance in monozygotic twins, that is, when both twins 1 and 2 have neuroblastoma and present either simultaneously or at different times, has not yet been described in detail. Furthermore, neuroblastoma in monozygotic twins is of interest because of the resulting insights regarding tumorigenesis and/or metastasis. In the present study, we reported simultaneous-onset neuroblastoma in monozygotic twins. Furthermore, we assessed the

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mechanism of this shared pathology using a genetic analysis and a review of the pertinent literature.

1. Case report

1.1. Clinical features and outcomes

The patients were infant male twins born by cesarean delivery following a healthy pregnancy at 36 weeks' gestation to unrelated parents. During the gestational course, the twins did not undergo exposure to extrinsic factors that might affect neuroblastomas. At 4 months of age, twin 2 presented with hepatomegaly and a right suprarenal mass (Fig. 1A-D). The patient's urinary vanillylmandelic acid (339 μg/mg Cr) and homovanillic acid (447 μg/mg Cr) were markedly elevated. Metaiodobenzylguanidine (MIBG) scintigraphy showed positive findings in the patient's right adrenal tumor and multiple liver masses (Fig. 1B). An adrenal tumor resection and liver tumor biopsy were performed. Twin 1 had no symptoms at 4 months of age. A screening by abdominal ultrasonography and a computed tomographic scan showed multiple masses in the patient's liver, but no adrenal mass (Fig. 1C). The patient's urinary vanillylmandelic acid (15.9 μg/mg Cr) and homovanillic acid (23.4 μg/mg Cr) were not elevated. Metaiodobenzylguanidine scintigraphy showed positive findings in multiple liver masses (Fig. 1D). A laparoscopic-assisted biopsy was performed for the liver tumor. All specimens of the primary adrenal tumor and liver tumor of twin 2 and the liver tumor of twin 1 exhibited favorable neuroblastoma regarding the Shimada classification [2] (Fig. 2A, B, C) and no MYCN amplification. According to these histology and biology of

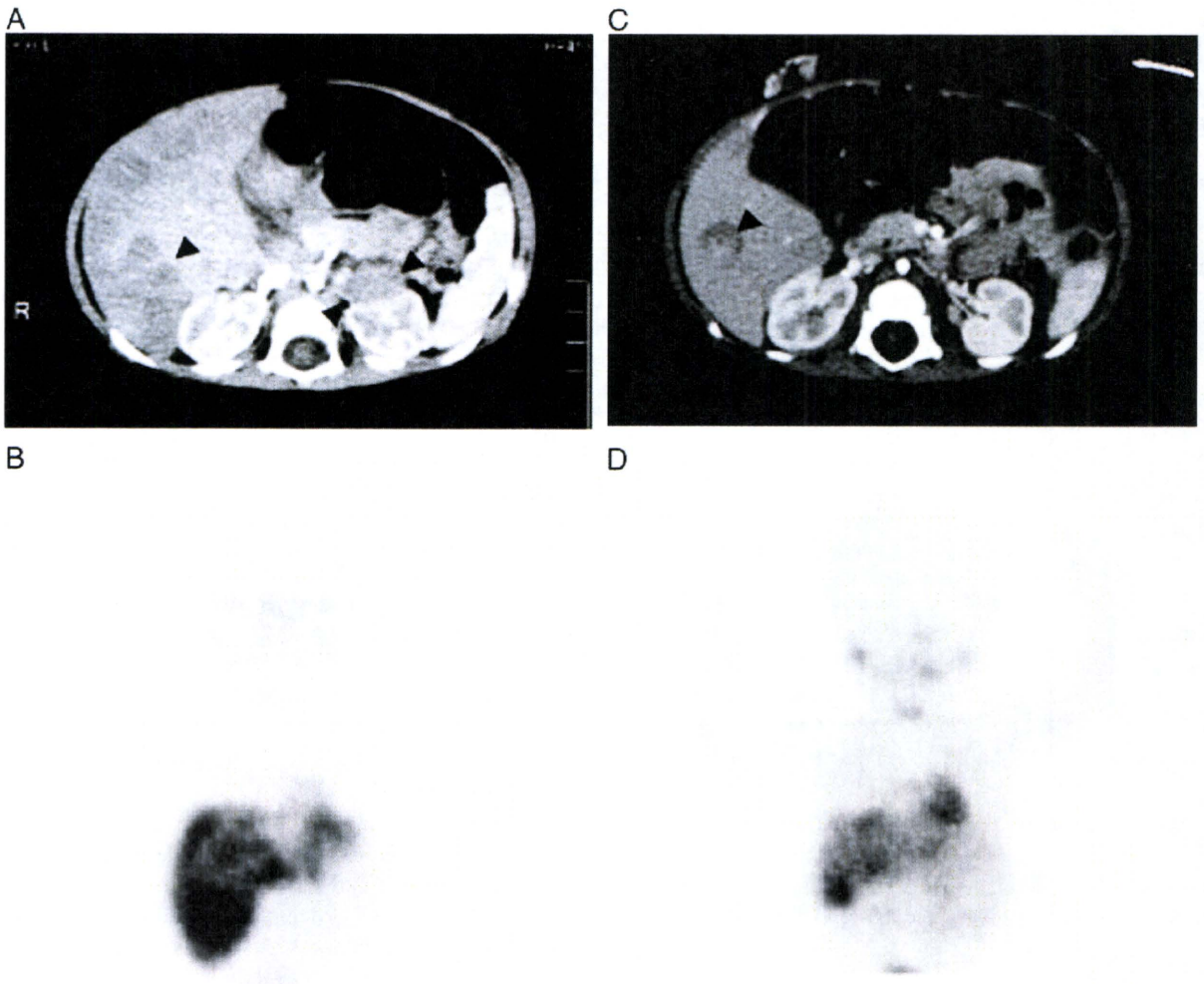

Fig. 1 Computed tomographic scan and MIBG scintigraphy of twin. A, Hepatomegaly and a right suprarenal mass in twin 2. B, Positive findings at right adrenal tumor and multiple liver masses in twin 2 as analyzed by MIBG scintigraphy. C, Multiple masses in liver, but no adrenal masses in twin 1. D, Positive finding at multiple liver masses in twin 1 using MIBG scintigraphy.

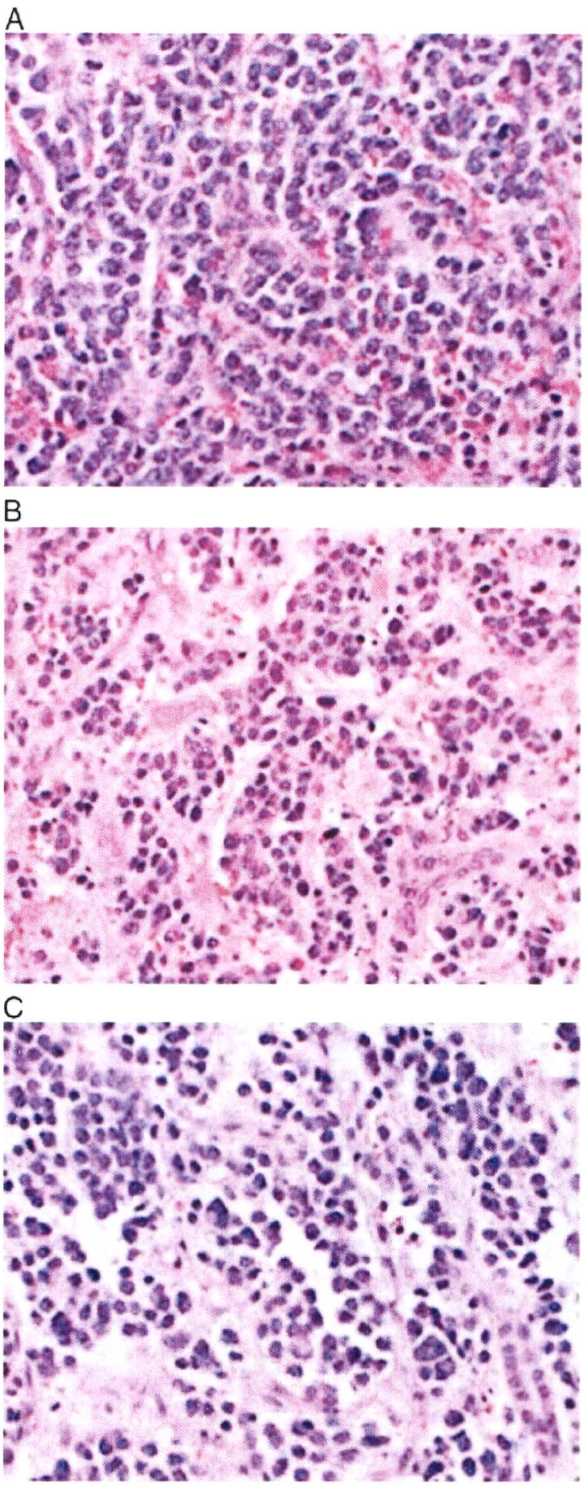

Fig. 2 Histology of the primary adrenal and liver tumors of twin 2 and the liver tumor of twin 1. A, Primary adrenal tumor of twin 2 (hematoxylin and eosin [H&E] stain). B, Liver tumor of twin 2 (H&E stain). C, Liver tumor of twin 1 (H&E stain). All sections show a proliferation of rounded cells with hyperchromatic nuclei and scant cytoplasm in a neuropil background, with histopathologic similarities at 3 sites.

tumors, mild chemotherapy including low dose of cyclophosphamide and vincristine was administered to twin 2 for a period of 6 months and patient observation is continuing for twin 1 for a period of 6 months after surgical intervention in both cases, respectively.

1.2. Histologic and genetic analysis for specimens of the primary tumor and the liver tumor of twin 2 and the liver tumor of twin 1

All specimens of primary adrenal tumor and liver tumor of twin 2 and liver tumor of twin 1 were indicative of a poorly differentiated neuroblastoma and also demonstrated a favorable histology regarding the Shimada classification. A histologic examination of several blocks of placenta revealed no metastatic tumor thrombi in villous stem vessels. No specimens had MYCN amplification, and all specimens were triploid.

To evaluate whole-genome aberrations, a microarray-based comparative genomic hybridization (array-CGH) was performed according to the methods described in a previous report by Tomioka et al [3] (244K Human CGH Oligo Microarray, Agilent Technology). For all specimens, the CGH type revealed entire chromosomal gains and losses type [3], which is associated with a favorable prognosis. Regarding the whole genome aberrations such as chromosome gains or losses, the only difference between the primary adrenal tumor and the liver tumor of twin 2 was chromosome 2; and the only difference between the primary adrenal tumor of twin 2 and the liver tumor of twin 1 was chromosome 14. Regarding chromosome 2, the primary adrenal tumor of twin 2 had a 2p whole gain and a 2q whole gain, whereas the liver tumor of twin 2 had a 2p partial gain and a 2q partial gain. Chromosome 14 was unchanged in the primary adrenal tumor of twin 2, but there was a 14q whole loss in the liver tumor of twin 1.

The consents of patients' parents for tumor preservation and the biological analysis were obtained before surgery. This study was performed according to the Ethical Guidelines for Clinical Research published by the Ministry of Health, Labor, and Welfare of Japan on July 30, 2003.

2. Discussion

Neuroblastomas can be multifocal and can be concordant or discordant (only one of the twins has the tumor) among monozygotic twins. To the best of our knowledge, as shown in Table 1, only 9 sets of monozygotic twins concordant for neuroblastoma, including the current cases, have been described; and no concordant neuroblastoma cases have been described for dizygotic twins [4-11].

Neuroblastomas can present in multiple ways. The most frequent presentation occurs in a child with a primary tumor (abdominothoracic) with or without metastases. In 1972,

Table 1 Review of literature of monozygotic twins concordant for neuroblastoma

References	Case	Sex	Age	Primary	Metastasis	Outcome	Zygosity
Lee, 1953	1	M	4 mo	L adrenal	Liver, bone, brain, testis	DOD 8.5 mo	Monozygous
	2	M	4 mo	R adrenal	Liver, bone, brain, testis	AWD 20 mo	
Cochran, 1963	3	F	–	R sympathetic	Liver	DOD 9.5 mo	Monozygous
	4	F	–	R sympathetic	Liver	DOD 11 mo	
Barrett and	5	–	Infancy	–	–	–	Identical twin
Toye, 1963	6	–	Infancy	–	–	–	
Miller, 1971	7	M	–	–	–	DOD 13 d	Twins, male
	8	M	–	–	–	DOD 16 mo	
Mancini, 1982	9	M	Birth	L adrenal	Liver, r adrenal medulla	Stillbirth	Identical twin and
	10	M	2 mo	None found	Liver	DOD 13 mo	single placenta
Boyd, 1995	11	M	1 wk	Retroperitoneum	Liver	DOD 1wk	*Diamnionic
	12	M	2 wk	None found	Liver, bone, skin, bil adrenal medulla	DOD 1 mo	monochorionic placenta
J Anderson, 2001	13	F	3 wk	L adrenal	Liver, bone marrow	NED 18 mo	Monozygous
	14	F	6 mo	R adrenal	Liver, r adrenal medulla	NED 18 mo	
I Adaletli, 2006	15	F	2 mo	L adrenal	Liver	NED 16 mo	Monozygous
	16	F	2 mo	None found	Liver	NED 16 mo	
Tajiri, (current cases)	17	M	4 mo	None found	Liver	Ongoing mild chemotherapy 6 mo	Monozygous
	18	M	4 mo	L adrenal	Liver	Ongoing observation 6 mo	

DOD indicates died of disease; AWD, alive with disease; NED, no evidence of disease.

* Diamnionic monochorionic placenta: Histopathologic examination of metastatic placental tumors revealed tumor thrombi in the villous stem vessels and terminal villi without gross macroscopic placental lesions.

Knudson and Strong [12] suggested 2 hypotheses that are the most widely accepted etiologic model for childhood neuroblastoma. The hypotheses invoke both inherited and acquired genetic defects as the basis for tumor development. In familial (hereditary) cases of neuroblastoma, the first defect is an inherited germ line mutation present in all cells of the body. A second defect or hit occurs postzygotically in only somatic target cells, the neuroblast. In sporadic (nonhereditary) cases, mutations are postzygotic events in the same neuroblast. The pathogenesis of multifocal neuroblastoma appears to occur through the multicentric growth of neuroblastoma nodules or neuroblastoma in situ with a potential for regression or maturation [13].

The cause of the shared pathology of concordance for neuroblastoma in monozygotic twins has not been well established. The question remains whether the disease is a simultaneous onset of malignancy in both twins or is because of metastatic spread via placental vascular anastomoses in utero from one twin with congenital disease to the second twin. Only monochorionic placentas share fetoplacental circulation and thus are a potential mechanism for metastasis. In cases 11 and 12 in Table 1, the histopathologic examination of metastatic placental tumors revealed tumor thrombi in villous stem vessels and terminal villi without gross macroscopic placental lesions [9]. The massive hepatic metastases could have arisen as a first-pass effect through the entrance of blood through the umbilical vein. Systemic metastases could also occur if an umbilical venous flow with tumor cells enters the right atrium. Fetoplacental metastases are favored in cases in which one twin within a twin pair manifests a readily identifiable primary tumor and twin 2 manifests the disease either simultaneously or later without a recognizable primary site. When both twins of a given twin pair display obvious primary tumors with similar disease extents, a diagnosis of simultaneous primary neuroblastoma is favored. In view of these clinical features, 5 pairs (cases 9, 10; cases 11, 12; cases 13, 14; cases 15, 16; and cases 17, 18) of 9 pairs of concordant twin neuroblastoma, as shown in Table 1, are considered to be placental metastases from one twin with congenital neuroblastoma to the other.

With the introduction of molecular and genetic markers such as MYCN and DNA ploidy, clear-cut biological markers are currently available to aid in differentiating simultaneous primary tumors from the metastatic spread between twins. However, the detailed whole genome analysis of tumor samples from monozygotic twins had never been done in the previous reports. When the primary neuroblastoma generates in one of a twin, a variety of chromosomal changes occur in the primary tumor. We have identified a variety of chromosomal changes in primary neuroblastomas using array-CGH or SNP array [3]. Afterward, a little chromosomal change may occur in the metastatic tumors, when neuroblastoma cells spread to other organs. In the present study, the adrenal primary adrenal tumor of twin 2 had a variety of genetic aberrations

including the gain or loss of chromosomes as analyzed by array-CGH, whereas the liver tumor of twin 2 and the liver tumor of twin 1 had only one genetic change from primary adrenal tumor of twin 2 using array-CGH, respectively. This result of genetic analysis suggests that placental metastases from one twin with congenital neuroblastoma to the other has occurred in the present cases.

In summary, in the current cases, twin 1 presents the disease simultaneously without a recognizable primary site; and all primary tumor and liver tumor specimens from twin 2 and the liver tumor of twin 1 had the same histologic classification of neuroblastoma and nearly identical genetic aberrations, including a chromosome gain or loss using array-CGH. From these clinical and pathologic findings and genetic analyses, we herein strongly demonstrate the transplacental metastatic spread from twin 2 to twin 1 as the mechanism of shared neuroblastoma pathology. This article is the first report presenting the possibility of twin-to-twin metastasis in monozygotic twins with neuroblastoma by the analysis of clinical features and genetic aberrations.

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References

[1] Crist WM, Kun LE. Common solid tumors of childhood. N Engl J Med 1991;324:461-71.
[2] Shimada H, Chatten J, Newton Jr WA, et al. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 1984;73:405-16.
[3] Tomioka N, Oba S, Ohira M, et al. Novel risk stratification of patients with neuroblastoma by genomic signature, which is independent of molecular signature. Oncogene 2008;17(27):441-9.
[4] Lee M. The surgical significance of tumors in identical twins: a short review of the literature and a report of sympathicoblastoma occurring in monozygotic twins. Am Surg 1953;19:803-11.
[5] Cochran W. Neuroblastoma (sympathicoblastoma) in Northern Ireland: a review over a ten year period. Ulster Med J 1963;32:82-98.
[6] Barrett AF, Toye DKM. Sympathicoblastoma: radiological findings in forty-three cases. Clin Radiol 1963;14:33-42.
[7] Miller RW, Fraumeni JF, Hill JA. Neuroblastoma: epidemiologic approach to its origin. Am J Dis Child 1968;115:253-61.
[8] Mancini Af, Rosito P, Faldella G, et al. Neuroblastoma in a pair of identical twins. Med Pediatr Oncol 1982;10:45-51.
[9] Boyd KT, Schofield ED. Monozygotic twins concordant for congenital neuroblastoma: case report and review of the literature. Pediatr Pathool Lab Med 1995;15:931-40.
[10] Anderson J, Kempski H, Hill L, et al. Neuroblastoma in monozygotic twins. A case of probable twin-to-twin metastasis. Br J Cancer 2001; 85:493-6.
[11] Adaletli I, Kurugoglu S, Aki H, et al. Simultaneous presentation of congenital neuroblastoma in monozygotic twin: a case of possible twin-to-twin metastasis. AJR Am J Roentgenol 2006;186:1172-5.
[12] Knudson Jr AG, Strong LC. Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet 1972;24:514-32.
[13] Hiyama K, et al. Multifocal neuroblastoma: biologic behavior and surgical aspects. Cancer 2000;88:1955-63.