Table 2 Summary of five patients with CAEBV treated with RIC and allogeneic bone marrow transplantation

Pt						Regimens of bone marrow transplantation									Outcome
Age (years)	Disease onset (years)	EBV-positive cells	Clinical manifestations	EBV genome (copies/μg DNA)	Therapy before transplant	Conditioning regimen (RIC)	Donor	HLA matched	Blood type	Cell number (cells/kg)	GVHD prophylaxis	Engraftment (d)	Complete chimera (d)	EBV-specific CTLs	Complications
1 8 males	7	CD4+ T	Generalized rash lymphadenopathy liver dysfunction aneurysm of coronary artery–positive EBV DNA in CSF ADEM	1.3×10^5	Modified HLH 2004 protocol	Flu 30 mg/m^2 × 4 CY 750 mg/m^2 × 4 TBI 3Gy × 1	UR	Full	A→AB	1.97×10^8	FK506 sMTX	12	136	−→+	Remission (65 mo) None
2 8 males	8	CD4+ T	Lymphadenopathy liver dysfunction positive EBV DNA in CSF	2.0×10^5	No	Flu 30 mg/m^2 × 4 CY 820 mg/m^2 × 4 TBI 3Gy × 1	Sibling	Full	A→AB	5.73×10^8	CsA sMTX	11	75	+→+	EBV-positive donor CD4+ T cells (60 mo) Acute GVHD grade II (gut) → steroid therapy → improved
3 21 females	8	NK	Lymphadenopathy mosquito allergy liver dysfunction hyper IgE	1.6×10^5	No	Flu 30 mg/m^2 × 5 CY 60 mg/kg × 2 TBI 2Gy × 2	UR	DR 1 locus mismatched	A→O	1.16×10^8	FK506 sMTX	19	168	+→+	Remission (55 mo) Acute GVHD grade I (skin), thrombocytopenia, hemolytic anemia → steroid + γ-globulin → improved
4 19 males	13	NK	Lymphadenopathy EBV-HLH liver dysfunction hyper IgE AR	2.1×10^5	No	Flu 30 mg/m^2 × 5 CY 30 mg/kg × 4 TBI 2Gy × 2	UR	Full	A→O	3.67×10^8	FK506 sMTX	21	129	−→+	Remission (51 mo) Acquired pure red cell aplasia → rituximab → improved
5 15 males	6	$\gamma\delta$ T	Hydroa vacciniforme liver dysfunction	1.2×10^5	No	Flu 30 mg/m^2 × 5 CY 60 mg/kg × 2 TBI 2Gy × 2	Sibling	Full	AB→AB	3.30×10^8	CsA sMTX	14	161	+→+	Remission (16 mo) Mixed chimera → donor leukocyte infusion → improved

Pt, patient; NK, natural killer; CSF, cerebrospinal fluid; ADEM, acute disseminated encephalomyelitis; AR, aortic valve regurgitation; HLH, hemophagocytic lymphohistiocytosis; Flu, fludarabine; CY, cyclophosphamide; TBI, total-body irradiation; UR, unrelated donor; CsA, cyclosporine A; FK506, tacrolimus; sMTX, short-term methotrexate; CTL, cytotoxic T lymphocyte; GVHD, graft-versus-host disease; mo, months.

marrow transplantation, types of donors, number of transplanted nucleated cells, the day of engraftment and achievement of complete chimera, EBV-specific CTLs, complications, and final outcome are summarized in Table 2. Patient 1 received a cooling therapy in HLH-2004 protocol consisted of cyclosporine A, dexamethasone, and etoposide before HSCT because he had several symptoms of hypercytokinemia. Other four patients received no pretransplant chemotherapy, because general condition was stable and pretransplant chemotherapy might increase the risk of infection and severe adverse effects before BMT. Patient 2 developed persistent EBV infection in donor T cells as described below. His EBV load has been remained positive and ranged from 2×10^2 to 5×10^3 copies/μg DNA. Although he has sustained low copy number of EBV genome in T cells for 60 months after allogeneic HSCT, he has been doing well without clinical symptoms. Patient 3 developed gradeIgraft-versus-host disease (GVHD) of the skin, autoimmune thrombocytopenia, and hemolytic anemia on day +182 and was successfully treated with steroid and immunoglobulin therapy. Patient 4 suffered from pure red cell aplasia due to major blood type incompatibility, which was successfully treated with rituximab. Patient 5 was in mixed chimera on day +134 and then achieved complete chimera by one course of donor leukocyte infusion with 1×10^7/kg CD3-positive T cells from the same sibling donor. All five patients achieved complete donor chimera on day 75 to 168. EBV load rapidly decreased to normal levels, and no severe RRT was observed following HSCT. All patients are alive without long-term complications for more than 51 months in four patients and 16 months in one patient after allogeneic HSCT.

Monoclonal proliferation and activation of CD4+ Vβ3+ T cells in a patient after allogeneic HSCT

In patient 2, steroid therapy was started from day +64 for 14 days because gastrointestinal GVHD symptom deteriorated from day +56. His EBV load was 2.0×10^5 copies/μg DNA before BMT. Although EBV load decreased to undetectable levels on day +52, regular monitoring of EBV load revealed up to 1×10^3 copies/μg DNA on day +99. Southern blot analysis showed monoclonal expansion of EBV strain. The most possible interpretation was EBV infection of the donor T cells, because his HLA-matched sibling donor was serologically positive for EBV and EBV load was ranged from 20 copies/μg DNA to undetectable levels. TCR Vβ spectrogram in donor T cells showed normal distribution (data not shown). However, TCR repertoire analysis showed selective proliferation of CD4+ Vβ3+ T cells in PB from patient 2 after BMT (Fig. 1A). The percentage of CD4+ Vβ3+ cells was as high as 24% of total

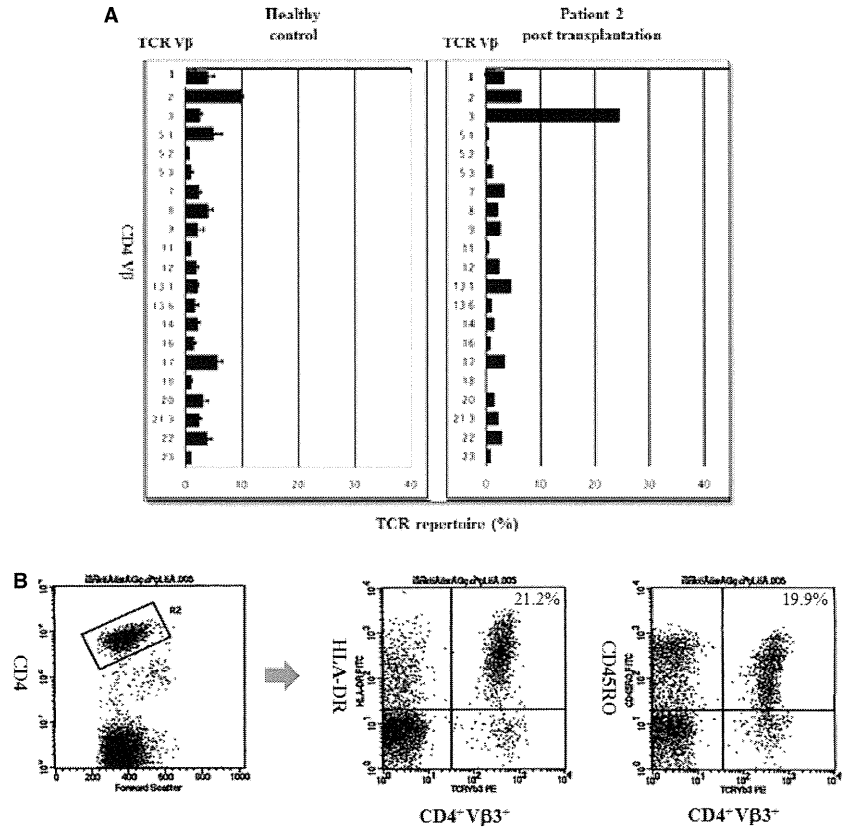

Figure 1 Monoclonal proliferation and activation of CD4+ Vβ3+ T cells in patient 2 after allogeneic HSCT. (A) Expression profile of TCR Vβ subfamilies. The percentages of each TCR Vβ-positive cells in total CD4+ T cells are shown in graph in healthy control (left) and patient 2 after transplantation (right). (B) FACS analysis of HLA-DR and CD45RO expression in CD4+ Vβ3+ T cells.

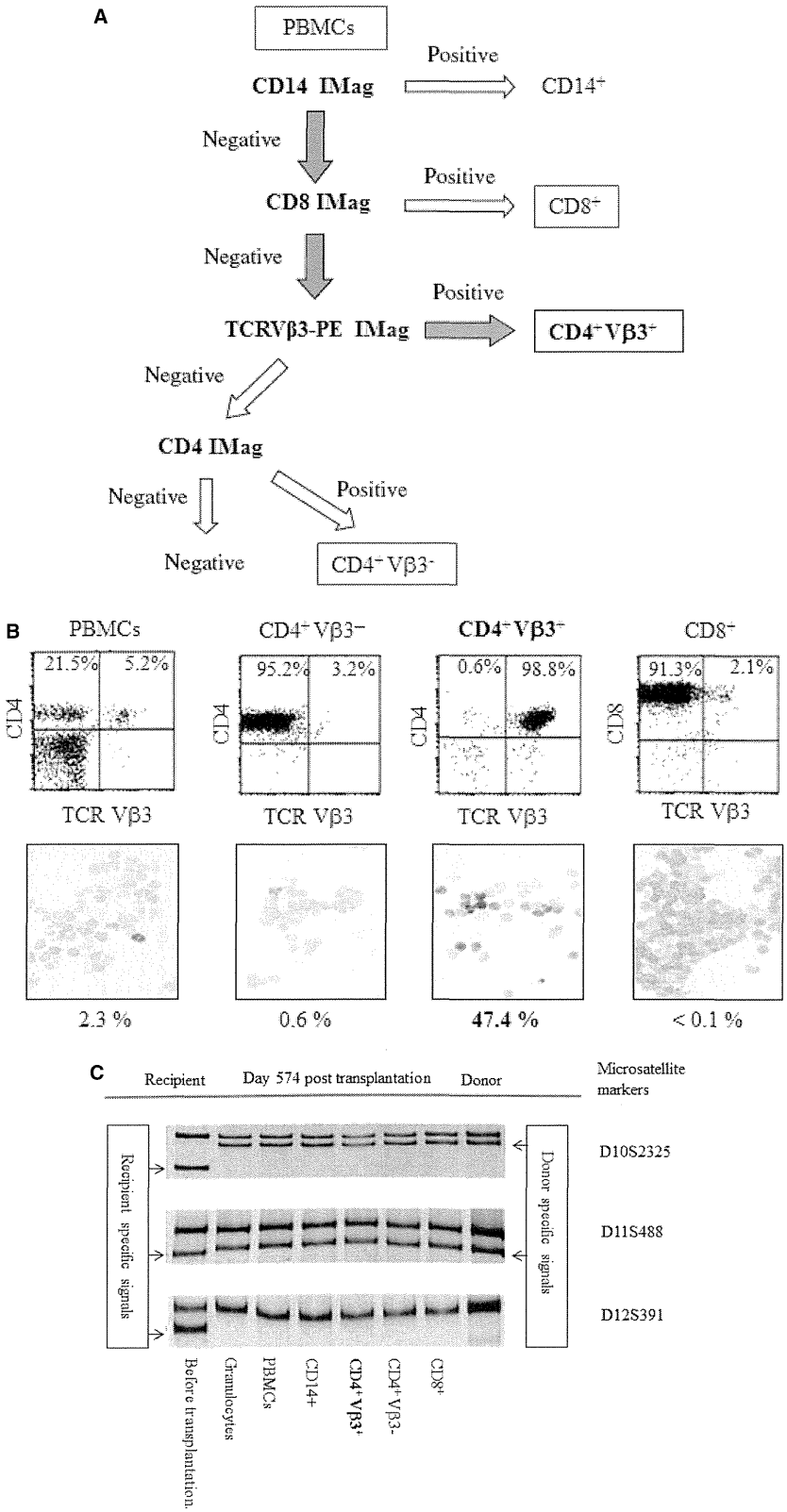

Figure 2 CD4+ Vβ3+ T cells in patient 2 after allogeneic HSCT selectively harbor EBV genome and are derived from donor T cells. (A) Separation of CD4+ Vβ3+ T cells from PBMCs by IMag method. (B) EBER-1 *in situ* hybridization shows that CD4+ Vβ3+ T cells are selectively EBER-1 positive. (C) EBV-infected CD4+ Vβ3+ T cells are derived from donor T cell. All fractions in PB on day 574 after transplantation and donor and recipient cells before transplantation are subjected to microsatellite analysis at three independent markers.

CD4+ T cells. Such clonal proliferation of CD4+ Vβ3+ T cells was not observed before HSCT. In addition, CD4+ Vβ3+ T cells exhibited high expression of HLA-DR and CD45RO, indicating most of the fraction was activated memory T cells (Fig. 1B).

CD4+ Vβ3+ T cells selectively harbor EBV genome and are derived from donor T cells

We next purified fractions of CD4+ Vβ3+ cells, CD4+ Vβ3- cells, and CD8+ cells from PBMCs by IMag methods (Fig. 2A). The purity of the separated CD4+ Vβ3+ cells was 98.8%, as determined by FACS analysis. 47.4% of CD4+ Vβ3+ cells were selectively EBER-1 positive determined by *in situ* hybridization, which was significantly higher than other CD4+ Vβ3- and CD8+ fractions (Fig. 2B). We next examined the origin of the EBV-infected T cells. Microsatellite analysis revealed that EBV-infected CD4+ Vβ3+ T cells and all other fractions were completely derived from donor cells (Fig. 2C).

Discussion

CAEBV is a chronic but fatal disease if patients are not treated with appropriate therapies. Recent controversial issues are when and how allogeneic HSCT should be performed and how we can reduce regimen-related toxicity (RRT) for better outcome. We selected RIC as a conditioning regimen consisted of Flu, CY, and low-dose TBI when the disease was in stable condition. Engraftment, complete chimera, rapid eradication of EBV genome, and no severe RRT were observed in all patients. We selected this regimen because we had successfully achieved the engraftment of donor stem cells without severe RRT in patients with primary immunodeficiencies (9, 10). We included low-dose TBI in the RIC regimen to strengthen immunosuppressive effects. It has been recently reported that RIC regimen resulted in significantly better outcome than myeloablative conditioning (8), consistent with our results that allogeneic HSCT with RIC was safer and curative treatment for patients with CAEBV.

Although pretransplant EBV loads were high in all patients, they rapidly reduced to normal levels after RIC and allogeneic HSCT. We did not treat the patients with pre-HSCT chemotherapy except patient 1, because we had no evidence that pre-HSCT chemotherapy was effective for achieving better outcome. Prior chemotherapy may decrease the EBV load in a part of the patients with CAEBV, and increased risk of infection and severe adverse effects should be considered before curative treatment of allogeneic BMT. It was also based on previous report to show that immunological effects of donor immune cells, rather than cytotoxic effects of chemotherapy, on EBV-infected cells have substantial roles in eradicating EBV genome following allogeneic HSCT (15).

We confirmed monoclonal proliferation of CD4+ Vβ3+ T cells in patient 2 after HSCT, and these unique cells were derived from donor T cells. At present, it is difficult to conclude whether engrafted donor T cells were newly infected by EBV in recipient or the donor CD4+ Vβ3+ T cells already infected by EBV before transplantation proliferated selectively after engraftment. We were unable to compare genomic sequences of EBV genome between recipient and donor cells before transplantation due to very low copy number of EBV genome in donor samples. We speculated that CD4+ Vβ3+ donor T cells expressing HLA-DR and CD45RO antigens were activated probably due to secondary activation event and escaped from immune surveillance after transplantation, which then clonally proliferated in the recipient. We may explain specific T-cell tropism rather than classical B-cell tropism in this patient 2 before and after BMT by similar genetic background of the recipient and his HLA-matched, EBV-seropositive sibling donor. Similar adult case of CAEBV was reported by Arai *et al.*, (16) who developed CAEBV again after allogeneic HSCT from an unrelated donor. In this report, EBV-infected cells were CD8-positive donor T cells and proliferated clonally. The infecting EBV strain in donor T cells differed from that before HSCT in the recipient. They suggested that host factors and immune suppression during the course of HSCT might be responsible for the development of donor-type CAEBV. In our patient 2, the donor was HLA-matched sibling donor who had genetic similarity to the recipient, and the patient received steroid administration before EBV load was elevated. However, further analysis is required to resolve this issue.

In conclusion, we report the safety and effectiveness of RIC and allogeneic HSCT in five patients with CAEBV in a single institute. To the best of our knowledge, patient 2 is the first pediatric patient in the literature that develops persistent EBV infection in donor T cells. Therefore, we have to consider the possibility of donor-derived EBV-infected cells as well as the relapse in recipient cells if EBV load increases after allogeneic HSCT.

Acknowledgements

We thank all of our colleagues in the Department of Pediatrics who have been involved in the patients' care and research.

Author contributions

YW, YS, and ST designed the research study, performed the research, analyzed the data, and wrote the manuscript. MS, TW, and AY performed the research. All other authors treated patients and collected clinical data.

Conflict of interest

The authors have no conflict of interest in the research and publication of this article.

Financial support

This work was supported by the grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20591242 and 23591528 to YS), a grant for research on intractable diseases from the Ministry of Health, Labour and Welfare of Japan (to YS), and grants from Japan Leukemia Research Fund and SENSHIN Medical Research Foundation (to YS).

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Cancer Science
The official journal of the Japanese Cancer Association

Heterogeneity of neuroblastoma cell lines in insulin-like growth factor 1 receptor/Akt pathway-mediated cell proliferative responses

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(Received December 6, 2012/Revised May 8, 2013/Accepted May 15, 2013/Accepted manuscript online May 27, 2013/Article first published online June 25, 2013)

Insulin-like growth factor 1 receptor (IGF-1R) is critical for cancer cell proliferation; however, recent clinical anti-IGF-1R trials did not show clear clinical benefit in cancer therapy. We hypothesized that IGF-1R signaling-mediated proliferative response is heterogeneous in neuroblastoma (NB) cells, and analyzed the cell growth of 31 NB cell lines cultured in three different media, including Hybridoma-SFM medium (with insulin) and RPMI1640 with/without 10% FBS. Three growth patterns were found. In response to IGF and insulin, cell proliferation and Akt phosphorylation were upregulated in 13 cell lines, and suppressed by MK2206 (Akt inhibitor) and picropodophyllin (IGF-1R inhibitor). Interestingly, 3 of these 13 cell lines showed Akt self-phosphorylation and cell proliferation in RPMI1640; their proliferation was downregulated by anti-IGF-1 or anti-IGF-2 neutralizing antibody, suggesting the existence of an autocrine loop in the IGF-1R/Akt pathway. Eighteen NB cell lines did not proliferate in RPMI1640, even though Akt phosphorylation was upregulated by IGF and insulin. Based on the heterogeneous response of the IGF-1R/Akt pathway, the 31 NB cell lines could be classified into group 1 (autocrine IGF-mediated), group 2 (exogenous IGF-mediated) and group 3 (partially exogenous IGF-mediated) NB cell lines. In addition, group 3 NB cell lines were different from group 1 and 2, in terms of serum starvation-induced caspase 3 cleavage and picropodophyllin-induced G2/M arrest. These results indicate that the response of the IGF-1R/Akt pathway is an important determinant of the sensitivity to IGF-1R antagonists in NB. To our knowledge, this is the first report describing heterogeneity in the IGF-1R/Akt-mediated proliferation of NB cells. (*Cancer Sci* 2013; 104: 1162–1171)

Neuroblastoma (NB), a malignant tumor that originates from the sympathetic nervous system, is one of the most frequent pediatric solid tumors.[1] NB is characterized by heterogeneous clinical behaviors, tumor invasiveness being different according to age and anatomic stage at diagnosis. The tumor is sometimes completely curable and may even regress spontaneously, especially in younger children.[2] Heterogeneity of the tumors depends on the degree of morphological differentiation and on histopathology.[3]

Insulin and insulin-like growth factors (IGF, including IGF-1 and 2) belong to a family of mitogenic growth factors. IGF, insulin, and their receptors are involved in normal growth and differentiation of most tissues. The biological actions of both IGF and insulin can be mediated by the IGF-1 receptor (IGF-1R), a transmembrane heterotetramer, which is involved in mitogenic, anti-apoptotic and oncogenic transforming responses.[4,5] The functional IGF-1R contains two extracellular α-subunits and two intracellular β-subunits that form a heterotetrameric complex. The structural homology of IGF-1R and insulin receptor (IR) allows formation of hybrid receptors (hybrid-R) in which an IGF-1R chain is connected to an IR chain.[6] Ligand interaction with α-subunits triggers the auto-phosphorylation of tyrosine kinase domains within the β-subunit.[7–9] The tyrosine kinase domains are coupled to several intracellular pathways, including the phosphatidylinositol-3-kinase-Akt (PI3K/Akt)[10,11] and the MAPK.[12] Dysregulation of the IGF-1R pathway is involved in promoting oncogenic transformation, cell proliferation, metastasis, angiogenesis and resistance in numerous malignant diseases, such as multiple myeloma,[13] carcinomas[14] and NB.[15] IGF-1R is also known to translocate to the nucleus to modulate gene expression.[16–18]

The IGF-1R inhibitors, including IGF-1R neutralizing antibodies, IGF-1 mimetics and IGF-1R anti-sense/siRNA, have been shown to block cancer cell proliferation.[19] Another target for treatment is the receptor tyrosine kinase (RTK).[20–24] The inhibitory effect of picropodophyllin (PPP) appears to be promising, because it has selectivity for the IGF-1R and, thus, lacks inhibitory activity on tyrosine phosphorylation of insulin RTK and other receptors, like fibroblast growth factor receptor, platelet-derived growth factor receptor and epidermal growth factor receptor.[25] Inhibition of the IGF-1 RTK with PPP is noncompetitive in relation to ATP, suggesting interference of the IGF-1R at substrate level.[23] It is reported that PPP specifically blocks phosphorylation of the Tyr1136 residue in the activation loop of IGF-1R kinase.[26] Inhibition of IGF-1R with PPP has been demonstrated in multiple myeloma,[23] breast cancer,[27] melanoma[28] and glioblastoma cells.[29]

Although IGF-1R and the stimulatory ligands (IGF and insulin) are important for cancer proliferation, anti-IGF-1R therapy has not shown enough clinical benefits in randomized phase III trials.[30] The mediation of IGF-1R signaling in cancer cell is still unclear.[30] We hypothesized that these unfavorable clinical results might be due to heterogeneity of IGF-1R signaling in cancer cells. The aim of the present study was to clarify the heterogeneous mediation of IGF-1R signaling in NB cell lines; for this purpose, we evaluated the cell proliferation patterns of 31 human NB cell lines by using three different media, stimulatory ligands and an IGF-1R inhibitor (PPP). The 31 NB cell lines were classified into three groups based on their differential response to the stimulatory ligands: group 1 (autocrine IGF-mediated), group 2 (exogenous IGF-mediated) and group 3 (partially exogenous IGF-mediated) NB cell lines. In addition, group 3 NB cell lines were different from groups 1 and 2 in terms of serum starvation-induced caspase 3 cleavage and

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doi: 10.1111/cas.12204

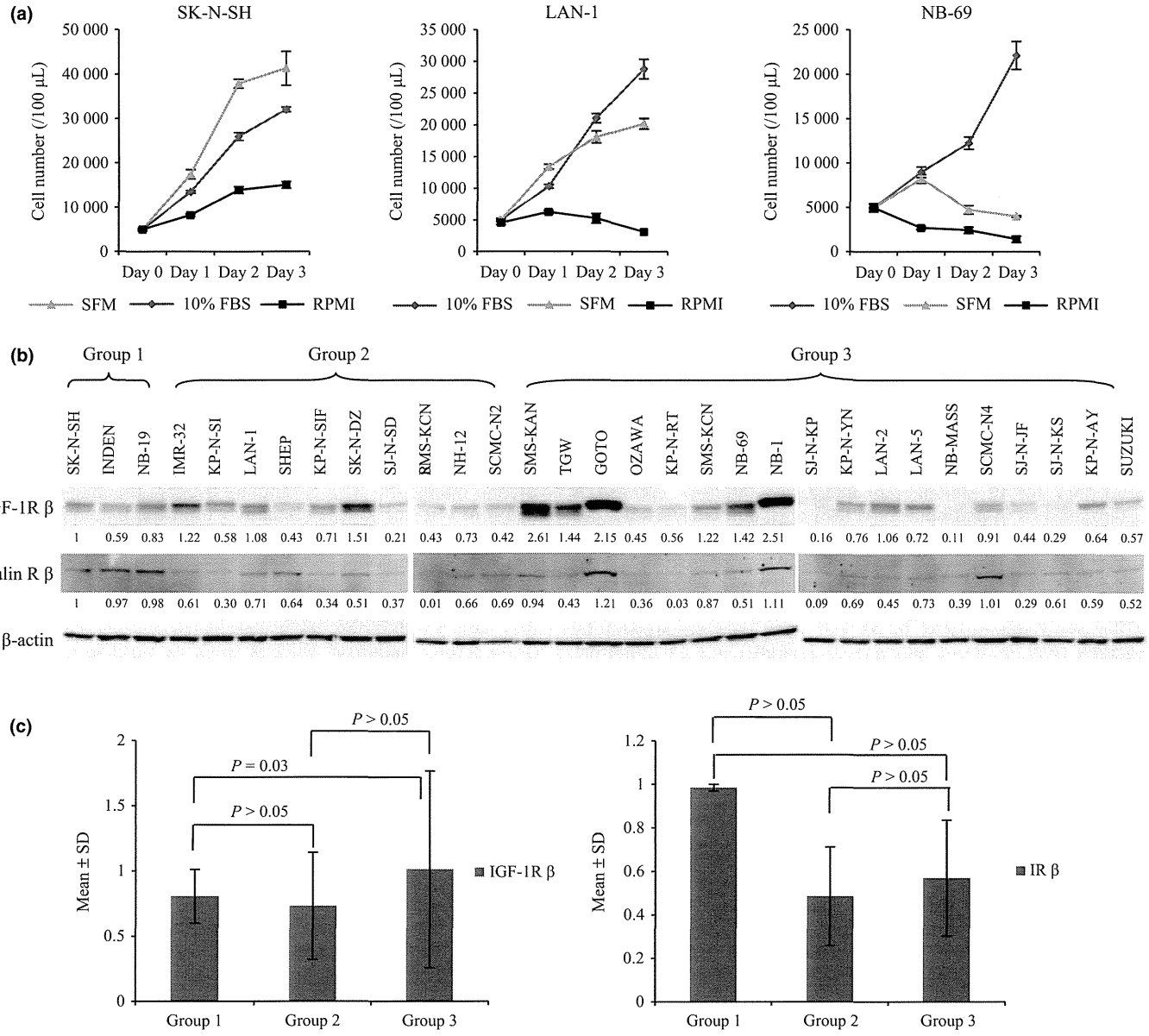

Fig. 1. The growth phenotype of the neuroblastoma (NB) cell lines in SFM and RPMI1640 with/without 10% FBS, and the expressions of insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR). (a) Three different growth patterns were shown by SK-N-SH, LAN-1 and NB-69 cell line in SFM and RPMI1640 with/without 10% FBS. (b) Expressions of IGF-1R and IR in 31 cell lines. Cell lines were cultured in RPMI1640 with 10% FBS, and lysed according to a previous report.[31] Target proteins were detected by anti-IGF-1R β antibody and anti-IR β antibody, with β-actin loaded as a control. Densitometric quantitation of IGF-1R β and IR β to β-actin ratio was standardized by the value of the first lane (SK-N-SH). (c) Bar graph shows densitometric analysis of the IGF-1R β and IR β subunit expression displayed according to the NB cell groups. Data are expressed as the mean (±SD) of the values showed in Figure 1b. Statistical analysis was performed using a two-sided *t*-test.

PPP-induced G2/M arrest. These results indicate that NB cell lines are heterogeneous in their IGF-1R-mediated signaling. The pattern of IGF-1R/Akt pathway-mediated proliferation is an important determinant of the response to IGF-1R antagonistic therapy in human NB. These observations suggest that IGF-1R/Akt pathway inhibitors, such as PPP and MK2206, may be used in NB clinical therapies.

Materials and Methods

Cell lines and cell culture. The following 31 human NB cell lines were used and evaluated in this study: SK-N-SH, INDEN, NB-19; IMR-32, KP-N-SI, LAN-1, SHEP, KP-N-SIFA, SK-N-DZ, SJ-N-SD, SMS-KCNR, NH-12, SCMC-N2; SMS-KAN, TGW, GOTO, OZAWA, KP-N-RT, SMS-KCN, NB-69, NB-1, SJ-N-KP, KP-N-YN, LAN-2, LAN-5, NB-MASS, SCMC-N4, SJ-N-JF, SJ-N-KS, KP-N-AY and SUZUKI. All these 31 cell lines were cultured in RPMI1640 (R8758, Sigma, St. Louis, MO, USA) medium supplemented with 10% FBS (GIBCO, Grand Island, NY, USA). The cells were incubated in a humidified atmosphere at 37°C with 5% CO_2. Thirteen of these cell lines could be cultured in SFM medium (Hybridoma-SFM medium, 12045-84; GIBCO), which contains low protein (20 μg /mL protein as insulin and transferrin).

Antibodies and reagents. The following antibodies and reagents were used in the present study: (i) rabbit monoclonal antibody: anti-Akt (#9272; Cell Signaling, Boston, MA, USA), anti-phospho-Akt (Ser473) (#4058; Cell Signaling); (ii) rabbit

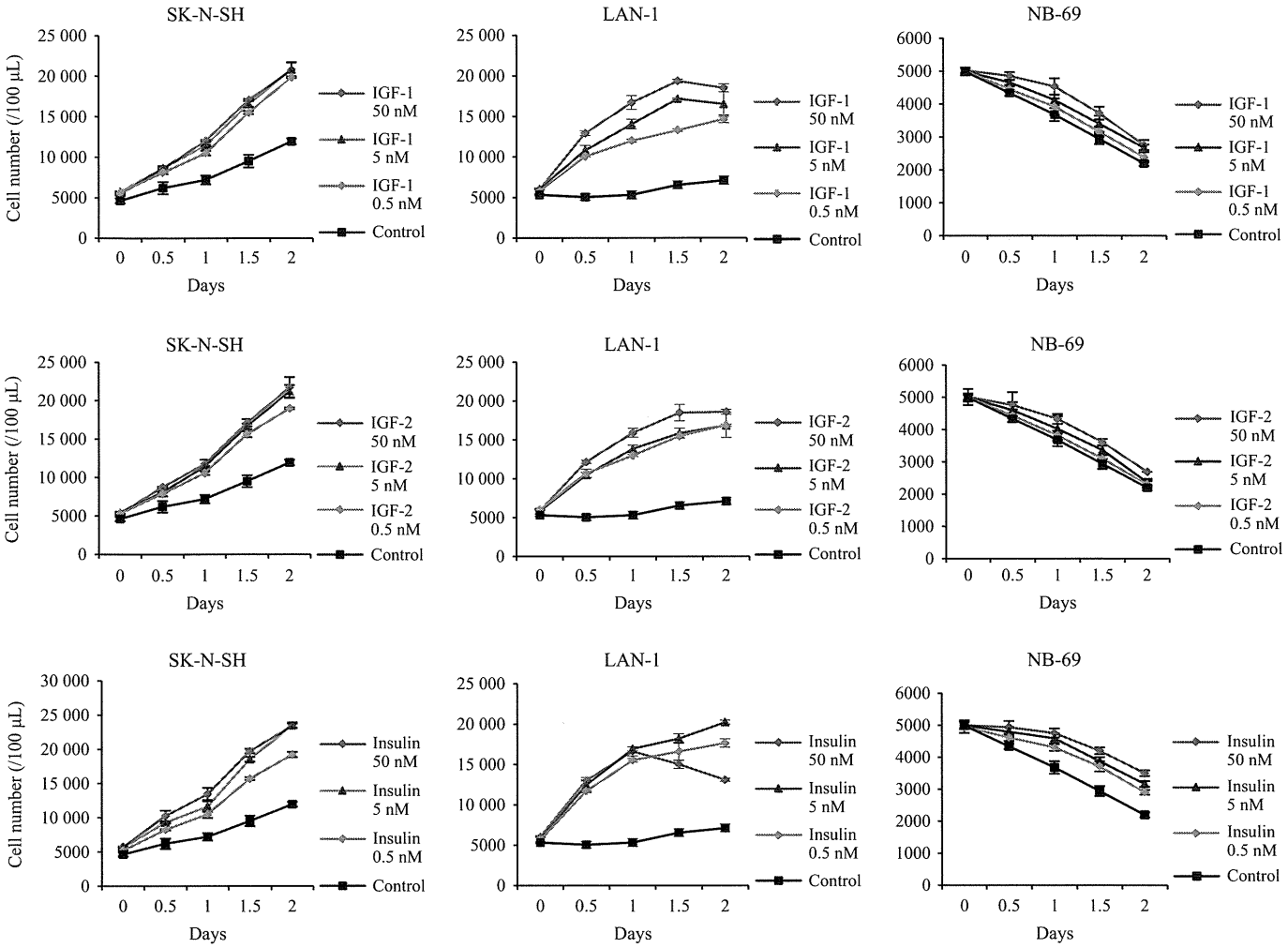

Fig. 2. The effect of exogenous stimulatory ligands on growth of neuroblastoma (NB) cell lines. SK-N-SH (group 1), LAN-1 (group 2) and NB-69 (group 3) cell lines were incubated in RPMI1640 with/without stimulatory ligands such as IGF-1, IGF-2 and insulin at the indicated concentrations. Proliferation of cell lines was evaluated as cell numbers at the indicated time points, and it was repeated three times. Data are expressed as the mean (±SD).

polyclonal antibody: anti-cleaved caspase 3 (Asp175) (#9661; Cell Signaling), anti-p44/42 MAPK (Erk1/2) (#9102; Cell Signaling), anti-IGF-1R β (#3027; Cell Signaling); (iii) mouse monoclonal antibody: anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9106; Cell Signaling), anti-cleaved PARP (Asp214) (#9546; Cell Signaling), anti-IR β (C-4) (sc-373975; Santa Cruz; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and (iv) neutralizing antibody: rabbit polyclonal IGF-1 neutralizing antibody (MAB791, R&B, Minneapolis, MN, USA), mouse monoclonal IGF-2 neutralizing antibody (MAB292, R&B).

Picropodophyllin (sc-204008) was purchased from Santa Cruz Biotechnology. MK2206 (Akt inhibitor, A10003) was purchased from Selleckchem (Houston, TX, USA). U0126 (MEK inhibitor, 70970-5) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell counting. WST-8 (Cell Counting Kit-8) cell counting reagent was obtained from Dojindo Molecular Technologies (Osaka, Japan). Cells (5×10^3) were seeded in 100 μL medium in 96-well plates and pre-incubated for 6 h before the addition of stimulatory ligands, inhibitors or neutralizing antibodies. WST-8 (10 μL) was added into each well at a 1:10 ratio in cell culture medium. After 2.5 h incubation in a humidified atmosphere at 37°C with 5% CO_2, the absorbance at 450 nm was measured using multi-spectrophotometer (Vienta, Dainippon, Japan). The optical density was then used to extrapolate the cell number from a standard curve. The standard curves were drawn for each cell line for each type of medium. The results are expressed as means ± SD from three independent experiments.

Western blotting. Cytoplasmic extracts were obtained as previously reported.[31] The proteins (40 μg/lane) were run on 7.5, 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by semi-dry transfer to PVDF membrane (Invitrogen, Carlsbad, CA, USA). Transferred PVDF blots were pretreated with 5% non-fat dry milk in TBST containing 0.1% Tween-20 and incubated with primary antibody (1:1000–3000) at 4°C overnight. The membrane was then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000–3000) for 1 h at room temperature. For phosphorylated target protein, transferred PVDF blots were pretreated with PVDF Blocking Reagent (TOYOBO, Osaka, Japan) for 1 h, and incubated with primary and then with secondary antibody (1:3000–6000), which were diluted with Can Get Signal Immunoreaction Enhancer Solution (TOYOBO) at room temperature for

doi: 10.1111/cas.12204

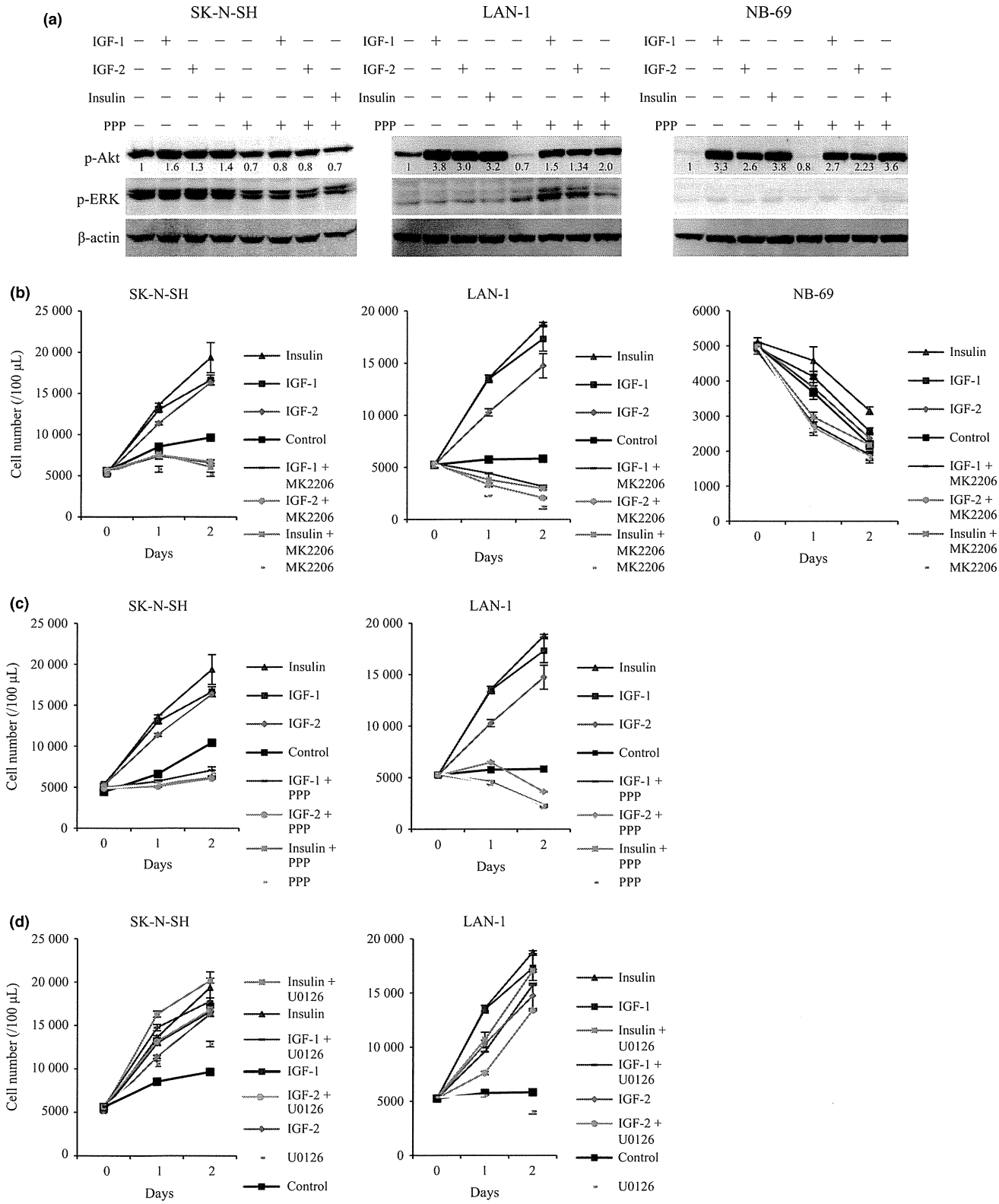

Fig. 3. Stimulatory ligands-induced phosphorylation of Akt and cell proliferation in RPMI1640. (a) Phosphorylation of Akt was induced by stimulatory ligands (IGF1, IGF-2, and insulin). The cell lines (SK-N-SH, LAN-1, NB-69) were pre-incubated with/without picropodophyllin (PPP) (2.5 μM) in RPMI1640 for 12 h, before 1 h stimulation with IGF-1 (5 nM), IGF-2 (5 nM), and insulin (5 nM). Phosphorylation of Akt was tested by WB, with the ratio of phospho-Akt compared with β-actin, and phospho-ERK. (b) MK2206 suppressed proliferation of neuroblastoma (NB) cell lines (SK-N-SH, LAN-1 and NB-69) incubated with stimulatory ligands. IGF-1 (5 nM), IGF-2 (5 nM), and insulin (5 nM) were co-incubated with cell lines with/without MK2206 (2.5 μM). (c) PPP suppressed NB (SK-N-SH and LAN-1) cell proliferation induced by stimulatory ligands. IGF-1 (5 nM), IGF-2 (5 nM) and insulin (5 nM) were co-incubated with cell lines with/without PPP (2.5 μM). (d) U0126 did not suppress NB cell proliferation induced by stimulatory ligands. IGF-1 (5 nM), IGF-2 (5 nM) and insulin (5 nM) were co-incubated with cell lines with/without U0126 (2.5 μM). Proliferation of the cell lines was evaluated as cell numbers at the indicated time points, and it was repeated three times.

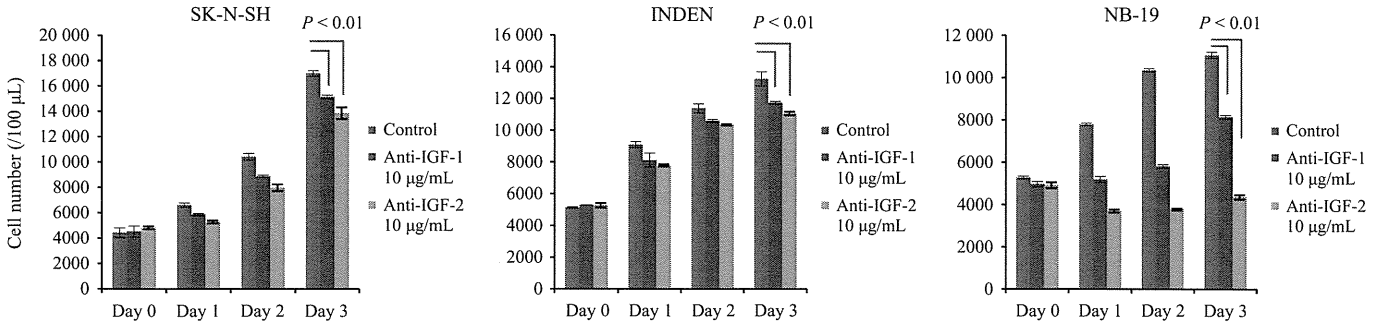

Fig. 4. Growth inhibitions of group 1 neuroblastoma (NB) cell lines induced by anti-IGF-1 or 2 neutralizing antibody. Group 1 NB cell lines (SK-N-SH, INDEN and NB-19) were cultured in the presence of anti-IGF-1 or IGF-2 neutralizing antibody (10 μg/mL) in RPMI1640. Proliferation of the cell lines was evaluated as cell numbers at the indicated time points, and it was repeated three times. Statistical analysis was performed using a two-sided *t*-test, $P < 0.01$, significant different.

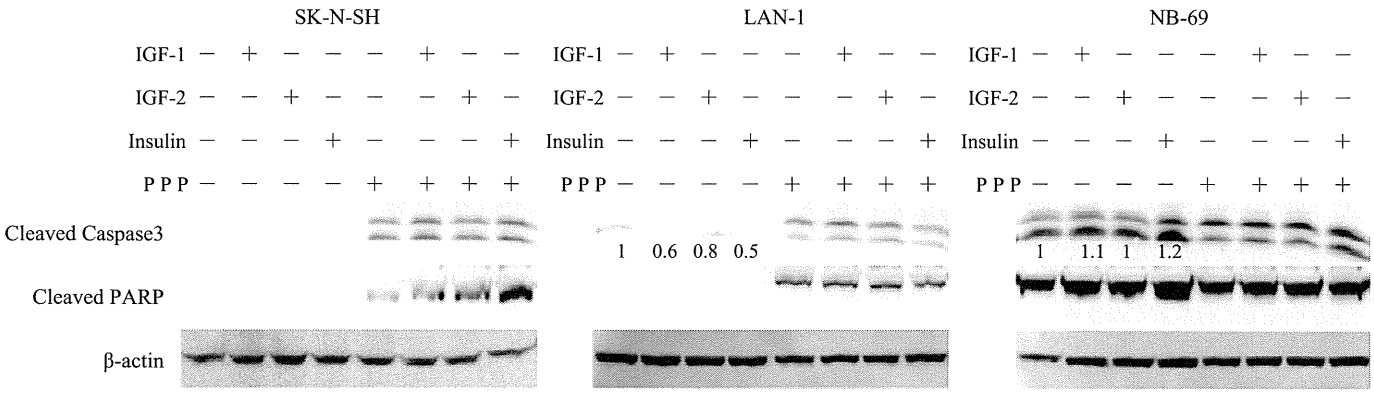

Fig. 5. Apoptosis of neuroblastoma (NB) cells induced by serum starvation or picropodophyllin (PPP). The indicated cell lines were pre-incubated in RPMI1640 with/without PPP (2.5 μM) for 12 h, before 1 h co-incubation with IGF-1 (5 nM), IGF-2 (5 nM) and insulin (5 nM). Cleavages of caspase 3 and PARP were analyzed by WB, and the ratio cleaved caspase 3 to β-actin was calculated.

1 h. After washing three times again, antibodies bound to protein blots were detected by using Western Lightening Chemiluminescence Reagent Plus (Perkin Elmer Life Science, Boston, MA, USA), visualized on LAS-3000 mini (FUJIFILM, Tokyo, Japan). The blots were quantitated and cropped using Multi Gauge Ver3.0 (FUJIFILM).

Cell cycle analysis. Cell cycle analysis was performed after treatment with/without PPP for 12 h. Cells (2×10^6) were harvested and fixed in 99.5% ethanol over night at −20°C, followed by incubation with 500 μL propidium iodide Triton X-100 solution containing RNase A at room temperature for 30 min in darkness, then the DNA content was analyzed immediately with a FACScan flow cytometer, using ModFitLT software (Verity Software House, Topsham, ME, USA).

Statistical analyses. A two-sided paired *t*-test was used to determine statistical significance. A $P < 0.05$ was considered as statistically significant.

Results

Sensitivity to culture conditions of neuroblastoma cell line groups. We hypothesized that NB cells are heterogeneous in their IGF-1R signaling-mediated cell proliferation. To test this hypothesis, 31 NB cell lines were cultured in three different media and screened using a cellular proliferation assay. Based on the response patterns we subdivided the 31 cell lines into three groups. Group 1, which included SK-N-SH, INDEN and NB-19, proliferated for more than 3 days in SFM (insulin containing), RPMI1640 (serum starvation medium) and RPMI1640 with 10% FBS (serum containing medium). Group 2 included IMR-32, KP-N-SI, LAN-1, SHEP, KP-N-SIFA, SK-N-DZ, SJ-N-SD, SMS-KCNR, NH-12 and SCMC-N2 cell lines; these cell lines could proliferate in SFM and RPMI1640 with 10% FBS but not in RPMI1640. Group 3 could proliferate only in RPMI1640 with 10% FBS. The growth curves of the three typical cell lines (SK-N-SH, LAN-1 and NB-69) in three different media are depicted in Figure 1(a). These results show that the regulation of cell proliferation induced by insulin is different among NB cell lines. Because the signal transduction of insulin is similar to IGF, these results indicate that signaling induced by IGF-1R stimulatory ligands may be different among NB cell lines.

Expressions of insulin-like growth factor 1 receptor and insulin receptor. Insulin-like growth factor 1 receptor is the common receptor of IGF and insulin.[6] Binding of IGF and insulin to the α-subunits of IGF-1R induces auto-phosphorylation of tyrosine kinase domain in β-subunit and phosphorylation of downstream signaling molecules. Expression of IGF-1R β-subunit and IR β-subunit were confirmed by western blotting using anti-IGF-1R β antibody and anti-IR β antibody. Both IGF-1R β-subunit and IR β-subunit were expressed in all 31 NB cell lines (Fig. 1b). Although the amounts of expressed β-subunits were not the same among NB cell lines (Fig. 1b), there was no significant statistical relationship between the receptor β subunit and the different groups of NB cell lines (Fig. 1c).

Neuroblastoma cell proliferation induced by exogenous insulin-like growth factor 1 receptor (IGF-1, IGF-2) and insulin. To confirm that NB cell proliferation is induced by stimulatory ligands, we evaluated NB cell proliferation in RPMI1640 in the presence of exogenous IGF (IGF-1, IGF-2) and insulin. These

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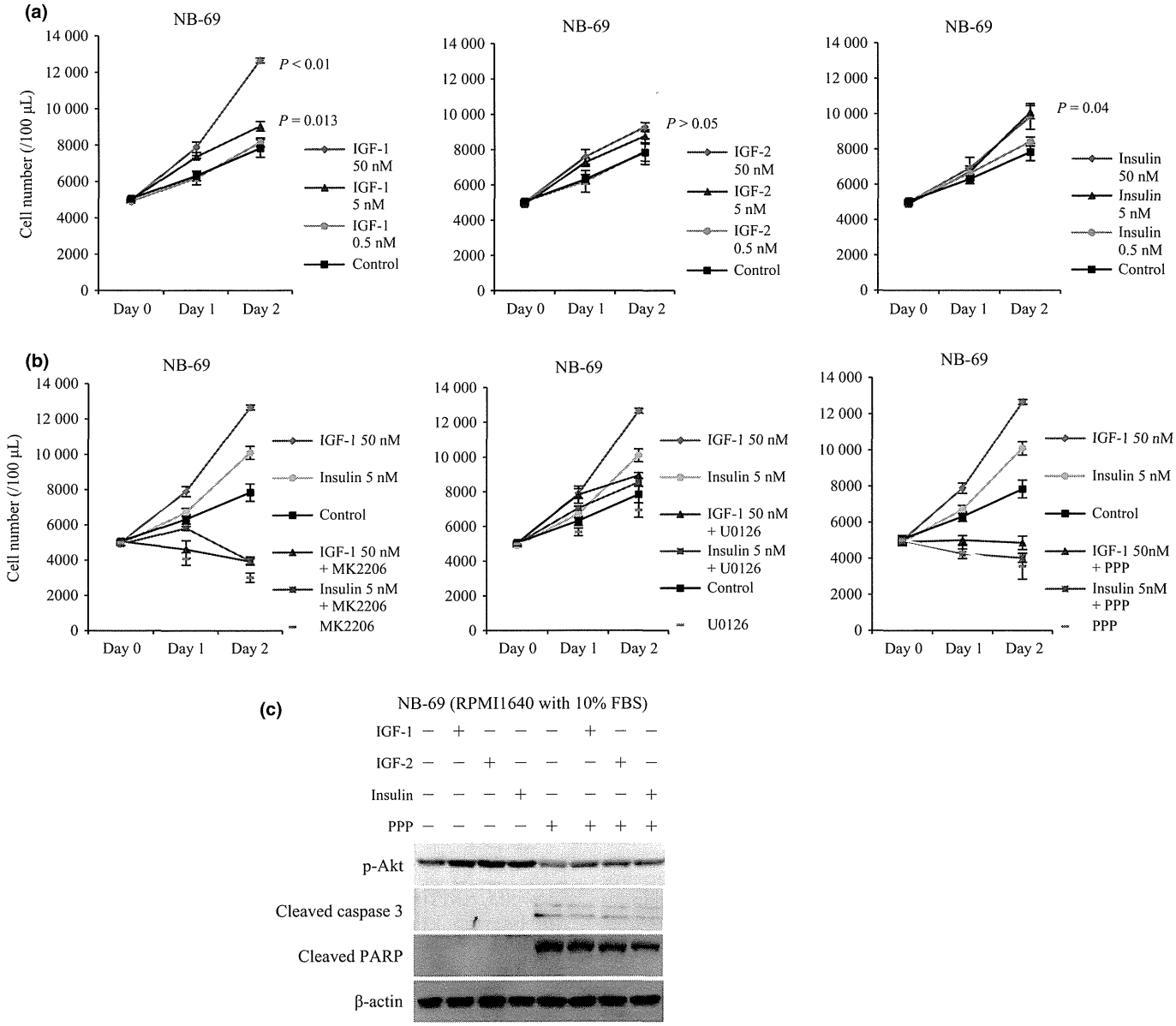

Fig. 6. The effect of exogenous IGF and insulin on NB-69 cell line in RPMI1640 with 10% FBS. (a) NB-69 cell line was incubated in RPMI1640 with 10% FBS with/without IGF-1, IGF-2, and insulin at the indicated concentrations. Proliferation of the cells was evaluated as cell numbers at the indicated time points. The experiment was repeated three times, and statistical analysis was performed using a two-sided *t*-test, $P < 0.01$, significant different. (b) NB-69 cell line subjected to MK2206 (10 μM), U0126 (10 μM) and picropodophyllin (PPP) (2.5 μM) with/without stimulatory ligands: IGF-1 (50 nM) and insulin (5 nM) in RPMI1640 with 10% FBS. (c) NB-69 cell line was pre-incubated with/without PPP (2.5 μM) in RPMI1640 with 10% FBS for 12 h, before 1 h stimulation of IGF-1 (5 nM), IGF-2 (5 nM) and insulin (5 nM). Akt phosphorylation, cleaved caspase 3, and cleaved PARP were evaluated by WB. β-actin was used as a control.

IGF and insulin accelerated cell proliferation in SK-N-SH (group 1) and LAN-1 (group 2) but not in NB-69 (group 3) (Fig. 2). These results suggest that IGF and insulin are important for cell proliferation of groups 1 and 2 NB cell lines, but not for that of group 3. SK-N-SH (group 1) was able to proliferate in RPMI1640 in the absence of exogenous IGF and insulin (Fig. 2). The other NB cell lines, group 1 (INDEN and NB-19), group 2 (SHEP, SMS-KCNR and KP-N-SI) and group 3 (OZAWA, KP-N-RT and SMS-KCN), showed the same response patterns of SK-N-SH (group 1), LAN-1 (group 2) and NB-69 (group 3), respectively.

Activation of insulin-like growth factor 1 receptor/Akt pathway induced by exogenous insulin-like growth factor 1 receptor (IGF-1, IGF-2) and insulin. Exogenous IGF and insulin induced Akt phosphorylation in SK-N-SH (group 1), LAN-1 (group 2) and NB-69 (group 3) cell lines (Fig. 3a). The Akt inhibitor, MK2206 (2.5 μM), completely inhibited Akt phosphorylation and cell proliferation induced by exogenous IGF and insulin in all 31 NB cell lines (data not shown). MK2206 strongly impaired the cell proliferation induced by exogenous IGF and insulin in SK-N-SH and LAN-1 cells (Fig. 3b). Interestingly, the IGF-1R inhibitor, PPP, inhibited Akt activation in SK-N-SH (group 1) and LAN-1 (group 2) cell lines, but less in NB-69 (group 3) (Fig. 3a). PPP suppressed the cell proliferation of SK-N-SH and LAN-1 (Fig. 3c).

Elevation of ERK phosphorylation was only observed in SK-N-SH (Fig. 3a). U0126, a MEK inhibitor, effectively suppressed ERK phosphorylation in NB cell lines (data not

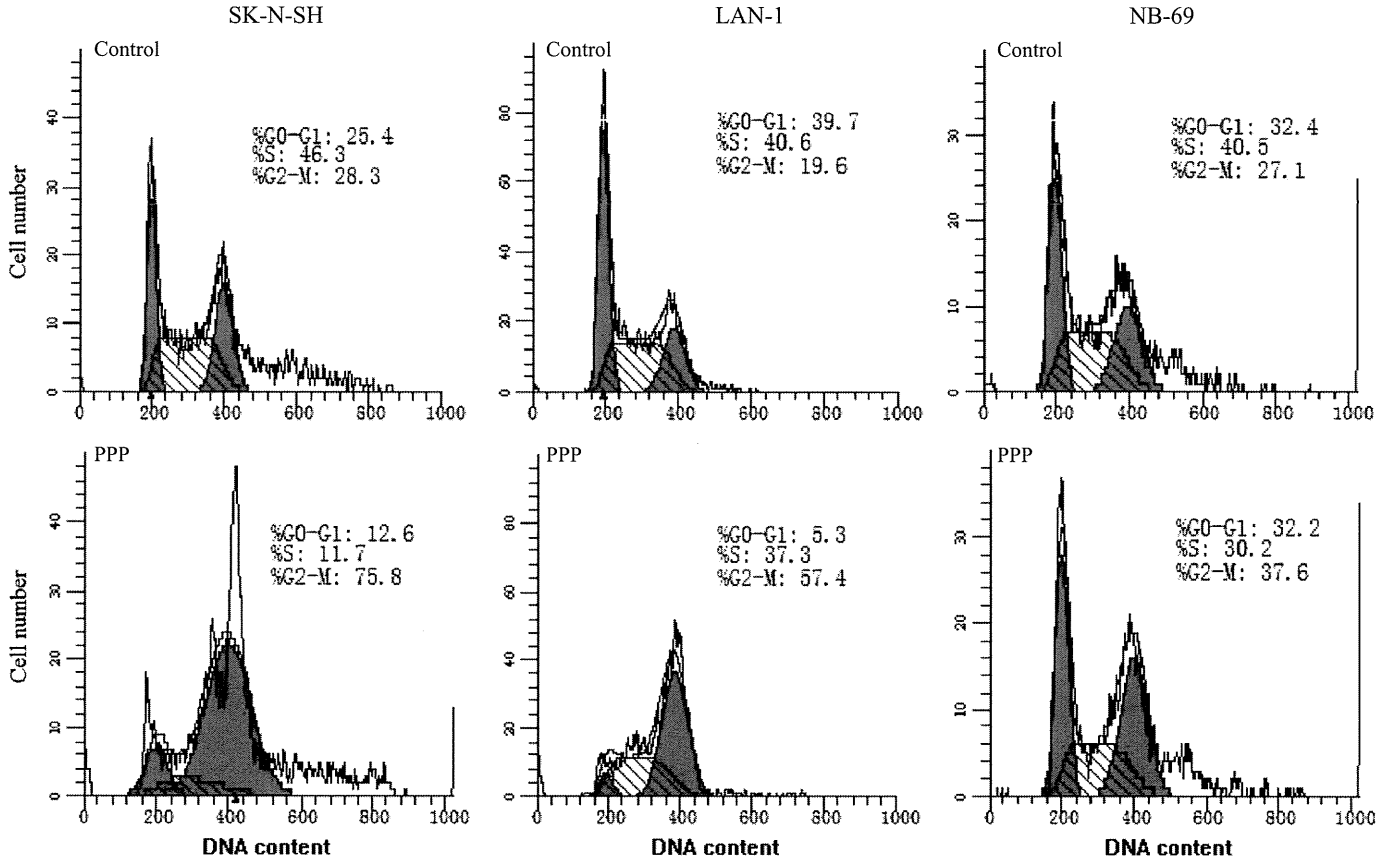

Fig. 7. The effect of picropodophyllin (PPP) on cell cycle phase distribution. The cell lines were treated with/without PPP (2.5 μM) for 12 h followed by analysis of cell cycle phase distribution in RPMI1640 with 10% FBS.[53] Cells were stained with propidium iodide for 30 min followed by FACScan flow cytometer.

shown). However, U0126 (2.5 μM) did not suppress SK-N-SH and LAN-1 cell proliferation induced by IGF (IGF-1, IGF-2) or insulin (Fig. 3d). These results suggest that IGF-1R/Akt pathway is critical for cell proliferation in group 1 and 2 NB cell lines. In group 3 NB cell line (NB-69), IGF and insulin activated IGF-1R/Akt pathway, but did not induce increased cell proliferation in RPMI1640, suggesting that the activation of IGF-1R/Akt pathway is insufficient for cell proliferation. The other NB cell lines, group 1 (INDEN and NB-19), group 2 (SHEP, SMS-KCNR, and KP-N-SI) and group 3 (OZAWA, KP-N-RT, and SMS-KCN), showed the same patterns of SK-N-SH (group 1), LAN-1 (group 2) and NB-69 (group 3), respectively.

Autocrine insulin-like growth factors in group 1 neuroblastoma cell lines. In group 1 NB cell lines (SK-N-SH, INDEN and NB-19), cell proliferation (Figs 1a,2) and Akt self-phosphorylation (Fig. 3a) were observed in RPMI1640 medium without exogenous IGF or insulin. The Akt self-phosphorylation was suppressed by PPP (Fig. 3a). This indirect evidence suggests the presence of an autocrine growth loop. Therefore, group 1 cell lines (SK-N-SH, INDEN and NB-19) were cultured in the presence of anti-IGF-1 neutralizing antibody and anti-IGF-2 neutralizing antibody. Both anti-IGF-1 and 2 neutralizing antibody impaired cell proliferation of group 1 cell lines in RPMI1640 medium (Fig. 4).

Apoptosis induced by serum starvation and insulin-like growth factor 1 receptor inhibitor. Insulin-like growth factors regulate apoptosis[32] and cell cycle progression.[33] Cleavages of caspase 3 and PARP were examined in NB cell lines. In group 1 (SK-N-SH), caspase 3 and PARP were not cleaved in RPMI1640 (Fig. 5). In group 2 (LAN-1), caspase 3 and PARP were cleaved in RPMI1640, and the cleavage of caspase 3 was suppressed by IGF and insulin (Fig. 5). Interestingly, in group 3 (NB-69), cleavages of caspase 3 and PARP were also observed in RPMI1640 (Fig. 5). However, they were not suppressed by IGF or insulin (Fig. 5), even though phosphorylated Akt was upregulated by IGF and insulin (Fig. 3a). In addition, in these NB cell lines, cleavages of caspase 3 and PARP were observed after treatment with PPP, regardless of the presence of exogenous IGF and insulin (Fig. 5).

Cell proliferation in group 3 neuroblastoma cell lines in the presence of insulin-like growth factor in RPMI1640 with FBS. Because group 3 NB cell lines died rapidly in a serum-deprived environment despite exogenous IGF or insulin (Figs 1a,2), Akt activation by IGF is insufficient to support cell proliferation and the proliferative stimulation may be induced by other growth factors present in FBS. Therefore, the effects of IGF, insulin, PPP, MK2206 and U0126 were evaluated on NB-69 incubated in RPMI1640 with 10% FBS. Cell proliferation of NB-69 was significantly accelerated by exogenous IGF-1 (50 nM) and insulin (5 nM) after 48 h (Fig. 6a). The cell proliferation was suppressed by MK2206 (10 μM) and PPP (2.5 μM), but not by U0126 (10 μM) (Fig. 6b). Cleavages of caspase 3 and PARP were suppressed in RPMI1640 with 10% FBS (Fig. 6c), and induced by the addition of PPP (Fig. 6c). These data suggest that the proliferation of group 3 cell lines depends on IGF-1R/Akt and on other not-yet-identified pathways.

G2/M-phase accumulation induced by insulin-like growth factor 1 inhibitor (picropodophyllin). We also analyzed cell cycle phase distribution in NB cells cultured in the presence of PPP, because PPP induced G2/M arrest and apoptosis by inhibiting

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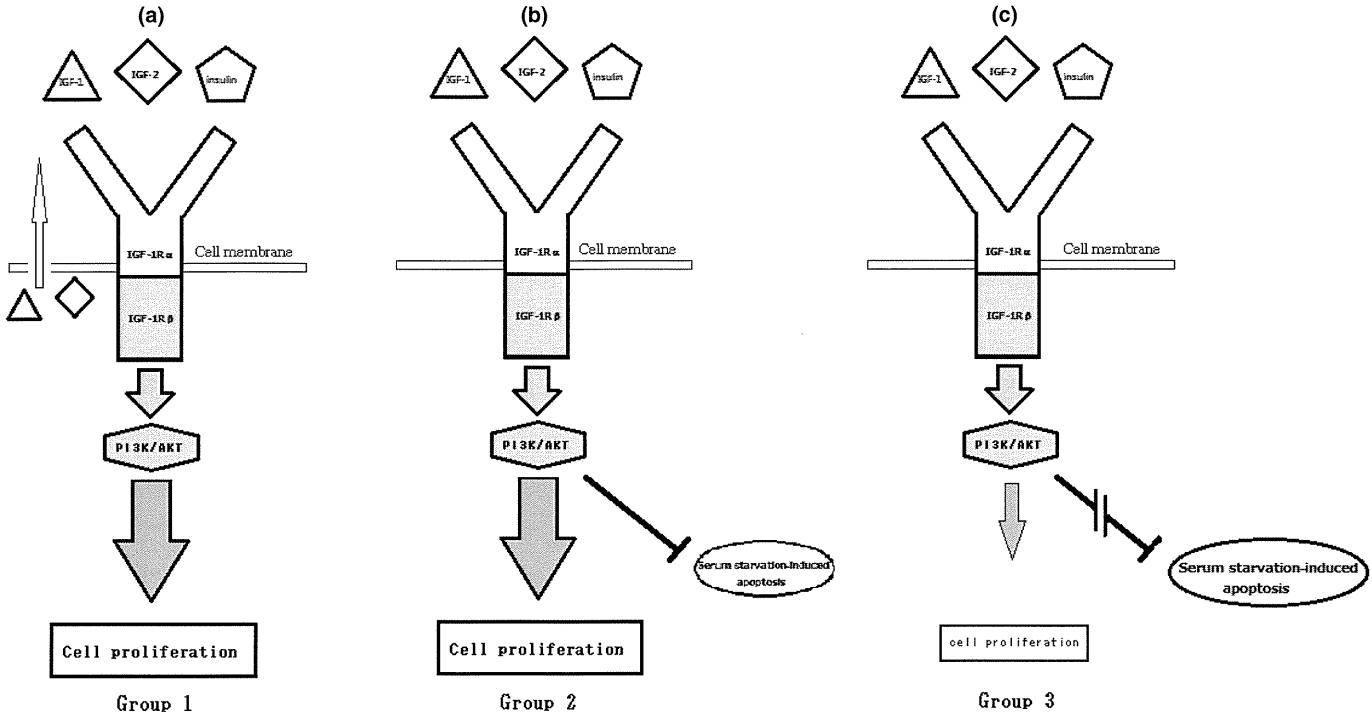

Fig. 8. Schematic pathways of insulin-like growth factor 1 receptor (IGF-1R)/Akt-mediated neuroblastoma (NB) cell proliferation in RPMI1640. (a) Proliferation of group 1 NB cell lines is mediated by IGF-1R/Akt pathway. IGF-1R/Akt pathway can be activated by IGF (autocrine or exogenous IGF) and insulin. (b) Proliferation of group 2 NB cell lines is mediated by IGF-1R/Akt pathway. Stimulation of IGF-1R/Akt pathway can be induced by exogenous stimulatory ligands such as IGF and insulin. Activated IGF-1R/Akt pathway suppresses serum starvation-induced apoptosis. (c) Activated IGF-1R/Akt pathway can hardly accelerate the cell proliferation of group 3 NB cell lines. Activated IGF-1R/Akt pathway cannot suppress serum starvation-induced apoptosis.

IGF-1R.[23,34] In our experiment, exposure to 2.5 μM PPP for 12 h increased the G2/M fraction of SK-N-SH and LAN-1 cell lines, and shifted cell cycle profile from G0-G1 dominant to G2/M dominant (Fig. 7). However, in the same condition, NB-69 did not show G2/M arrest, NB-69 only showed an elevation of G2/M from 27.1 to 37.6%, and the G0/G1 fraction was not affected (Fig. 7).

In a parallel experiment, we also found that, in SK-N-SH and LAN-1, cyclin B1, a marker protein of G2/M phase of cell cycle, was upregulated, and when the typical G2/M arrest occurred, cyclin D1, a marker protein of G0/G1, synchronously declined. In NB-69 (group 3), accumulation of cyclin B1 was duplicated by PPP, but cyclin D1 was not affected (data not shown). This may be explained by the insensitiveness of group 3 cell lines to PPP-induced G2/M arrest. The other NB cell lines, group 1 (INDEN and NB-19), group 2 (SHEP, SMS-KCNR, and KP-N-SI) and group 3 (OZAWA, KP-N-RT, and SMS-KCN), showed the same patterns of SK-N-SH (group 1), LAN-1 (group 2) and NB-69 (group 3), respectively.

Discussion

In this study, we demonstrated that NB cell lines are heterogeneous in terms of their IGF-1R/Akt pathway-mediated cell proliferation. The cells can be categorized into three different groups (Fig. 8). Group 1 cell lines (autocrine IGF-mediated cell lines) produce endogenous IGF; IGF-1R/Akt pathway is activated by both exogenous and autocrine IGF; these cell lines can proliferate for more than 3 days in RPMI1640 with 10% FBS, insulin containing SFM and even in RPMI1640. Group 2 cell lines (exogenous IGF-mediated cell lines) need the stimulation of exogenous stimulatory ligands, including IGF and insulin for cell proliferation; these cell lines can proliferate in SFM and RPMI1640 with 10% FBS, but not in RPMI1640. Group 3 cell lines (partially exogenous IGF-mediated cell lines) can proliferate in RPMI1640 with 10% FBS, and their proliferation is accelerated by IGF and insulin only in the presence of FBS. These observations suggest that group 3 NB cell proliferation is mediated by both IGF and not-yet-identified growth factor presence in FBS. To our knowledge, this is the first study describing heterogeneity in the IGF-1R /Akt pathway in NB cell lines. Subdivision of NB cell lines by their heterogeneity will help to further clarify the mechanism of IGF-1R mediation.

Insulin-like growth factor 1 receptor and insulin induced cell proliferation through Akt activation; blockage of IGF-1R suppressed Akt activation only in group 1 and 2 cell lines. However, the blockage of IGF-1R did not markedly affect Akt activation in group 3 cell lines cultured in RPMI1640. In contrast, the activation of Akt could not suppress serum starvation-induced apoptosis in the partially exogenous IGF-mediated NB cell lines (group 3). These findings may prompt reconsideration of cancer therapy targeting IGF-1R and IGF.[30] The anti-IGF-1R antibodies used in clinical trials, known as A12,[35] h7C10,[36] EM164[37] and CP-751, 871,[38] affect IGF-1R/Akt pathway, then cell apoptosis and cell proliferation. However, further *in vivo* experiments are required to support these observations.

Picropodophyllin, the IGF-1R inhibitor, was able to effectively inhibit the exogenous IGF-induced Akt phosphorylation in groups 1 and 2, but not in group 3. PPP blocks phosphorylation of tyrosine cluster Y1136 in IGF-1R,[26] which is the main binding site of insulin receptor substrate 1 (IRS-1).[39] IR substrate 2 (IRS-2) interacts with IGF-1R via multiple binding motifs,[40] independent of tyrosine cluster Y1136 in IGF-1R. Difference in IRS binding specificity may explain

these observations. Our data also showed that NVP-AEW541 (2.5 μM), a nonspecific IGF-1R RTK inhibitor, suppressed the IGF-1R/Akt pathway in all three groups (data not shown). These results indicate that the signal transduction from IGF-1R to PI3K/Akt complex may be different in NB cell line groups. Both IRS-1 and 2 are able to stimulate PI3K-Akt signal pathway.[41] IRS-1 is important for mitogenesis, cell proliferation and survival, whereas IRS-2 is important for adhesion, migration and metastasis.[6,42] We speculate that differences in binding of IRS to IGF-1R may lead to different IGF-1R/Akt-mediated signals. Selective knockdown or overexpression of IRS-1 or 2 are necessary to verify the relationship between IRS and IGF-1R stimulation.

Picropodophyllin interrupts DNA synthesis by inhibition of IGF-1R and inducing G2/M arrest.[43] G2/M-phase accumulation induced by PPP is due to interference with the CDK1/cyclin B1 complex.[23] Cyclin B1 protein begins to increase during G2, peaks in mitosis, and is rapidly degraded before the cell cycle is completed; it is a specific marker of G2/M.[44] Cyclin D1 is a G1-specific cyclin that associates with CDK4 or CDK6, and promotes restriction point progression during G1 phase.[45] Expression and accumulation of cyclin D1 occur at multiple levels, including increased transcription, translation and protein stability, and are affected by growth factors,[46] or signal pathways including PI3K-Akt pathway.[47] Ectopic expression of cyclin D1 induces G2/M arrest.[48] In response to PPP and MK2206, cyclin B1 accumulated in all three groups, but cyclin D1 was declined only in groups 1 and 2 NB cell lines, and not in group 3 (data not shown). These results indicate that IGF-1R/Akt pathway exerts less effect on cyclin D1 in group 3 NB cell lines, and this may explain why group 3 NB cell lines are insensitive to PPP-induced G2/M arrest.

Apoptosis and G2/M arrest induced by PPP were demonstrated in multiple myeloma cells[23] and in other cancer cells. Alternative signaling of IGF-1 and 2 may occur through other insulin-related receptors, like the IR[49] or heterodimerization of IGF-1R with other receptors such as the epidermal growth factor receptor.[50] These observations may explain the lack of efficacy of therapeutic antibodies since they exert no inhibitory activity on other receptors, as is the case for other receptor tyrosine kinase inhibitors.[51,52] Moreover, PPP has been shown to be well tolerated *in vivo* after oral administration.[25]

In summary, we showed in this study heterogeneity of the IGF-1R/Akt pathway in several NB cells lines. NB cell lines can be categorized into three groups by the patterns of IGF-1R/Akt pathway response. So far, the clinical and biological correlations of IGF-1R response in tumors are poorly understood.[6] Biomarkers are needed to predict clinical responses to IGF-1R antagonists, and to select patients who can be benefited from IGF-1R-targeted therapy. We speculate that comparative analysis of the response between different groups with/without stimulatory ligands by using microarray analysis may be helpful to find novel biomarkers. The mechanism of this heterogeneous response could be the topic of future studies.

Disclosure Statement

The authors have no conflict of interest.

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Gonadal function in patients with severe aplastic anaemia and refractory cytopenia of childhood who undergo bone marrow transplantation after receiving 3-Gy total body irradiation and high-dose cyclophosphamide

Fertility preservation has a significant influence on the quality of life of survivors of haematopoietic stem cell transplantation (SCT; Hammond *et al*, 2007). Several previous studies have reported on gonadal function and pregnancy after SCT in patients with severe aplastic anaemia (SAA), most of whom underwent matched related donor (MRD) bone marrow transplantation (BMT) after receiving 120–200 mg/kg cyclophosphamide (CY) with or without anti-thymocyte globulin (ATG) as a conditioning regimen (Sanders *et al*, 1996, 2011; Loren *et al*, 2011). Normal gonadal function was observed in most patients, and the probability of pregnancy in female patients or in the partners of male patients was reported to be approximately 50%. In recent years, the outcomes of matched unrelated donor (MUD) BMT, which is an alternative therapeutic option for SAA patients who do not have a MRD and fail one or more courses of immunosuppressive therapy, have improved owing to the introduction of high-resolution human leucocyte antigen (HLA) typing and the optimization of conditioning regimens, such as the addition of low-dose total body irradiation (TBI; Vassiliou *et al*, 2001; Deeg *et al*, 2006). The utilization of low-dose TBI as part of conditioning regimens has substantially increased; however, when low-dose TBI is used in combination with CY, the gonadotoxicity of the regimen is unclear. We analysed the fertility status of patients with SAA and refractory cytopenia of childhood (RCC) who underwent BMT after receiving 3 Gy TBI and 200 mg/kg CY-based conditioning regimen.

Twenty-three consecutive patients with SAA ($n = 18$) or hypocellular RCC ($n = 5$) underwent BMT at our centre between March 1986 and April 2011. Nineteen patients had survived for at least 1 year after BMT without disease relapse and were at least 12 years of age at the time of evaluation (Table I). TBI was delivered via a single fraction at a dose rate of 0·07 Gy/min on day −6. No shielding was used for the testes and ovaries. CY was administered at 50 mg/kg/d on days −5 to −2. For patients with an HLA-mismatched related donor (MMRD) or MUD, ATG (Zetbulin; Fresenius Biotech GmbH, Munich, Germany) was added to the regimen at 2·5 mg/kg/d on days −5 to −2. Donor selection, graft-*versus*-host disease prophylaxis, and other procedures for BMT were previously described (Inagaki *et al*, 2007).

Fertility status was evaluated using documented clinical information and laboratory data for gonadotropin and sex hormone concentrations. Elevated gonadotropin levels and decreased sex hormone levels were defined as follows: luteinizing hormone (LH) >15 u/l, follicle-stimulating hormone (FSH) >15 u/l, testosterone in male patients <6 nmol/l, and oestradiol in female patients <110 pmol/l (Borgmann-Staudt *et al*, 2012).

Median follow-up time was 120 months (range, 22–317 months), and none of the patients received hormone replacement therapy. Among 14 male patients, three patients had married and fathered a child at 12, 13, and 10 years after BMT, respectively (Table I). Another patient had a semen analysis 5 years after BMT and normal spermatogenesis was observed. Analyses of gonadotropin and sex hormone concentrations were performed in 13 male patients. A mild increase in the FSH level was observed in two patients, whereas a normal FSH level was observed in the remaining 11 patients. All male patients had normal LH levels. Two patients who had low testosterone levels required careful follow-up regarding the suspicion of hypothalamic/pituitary disorder.

Spontaneous menstruation occurred in all the five female patients at a median age of 12 years (range, 10–12 years) and continued until the last follow-up. The menstrual cycle was almost regular in all patients. An oestradiol level slightly lower than normal was observed in two patients without elevated gonadotropin levels (Table I).

As SAA patients generally do not receive cytotoxic agents or radiation before SCT and the conditioning regimens do not commonly include busulfan or TBI, gonadal function after SCT has been reported to be less impaired. The present study showed that gonadotropin and sex hormone levels were almost normal in most of the evaluable patients who underwent BMT after 200 mg/kg CY and 3 Gy TBI. All of the five female patients had spontaneous menstruation after BMT, therefore it is possible that they may conceive in the future. Although pregnancy occurred in the partners of only three male patients, our study does not account for the proportion of patients who were actually trying to conceive. The fact that all three married

Table I. Patient characteristics and gonadal function.

Patient	Sex	Diagnosis	Donor	Conditioning regimen*	Age at BMT (years)	Age at evaluation	LH (u/l)	FSH (u/l)	Testosterone (nmol/l)	Oestradiol (pmol/l)	Fertility (age, years)
1	M	SAA	MRD	TBI+CY	3	30	8·3	20·4	12·7	–	–
2	M	SAA	MMRD	TBI+CY	10	14	2·5	2	1·3	–	–
3	M	SAA	MRD	TBI+CY	14	20	9·4	16·4	16·5	–	–
4	M	SAA	MMRD	TBI+CY+ATG	16	21	4·9	8	30·9	–	–
5	M	SAA	MRD	TBI+CY	2	19	3·8	6	12·1	–	–
6	M	SAA	MRD	TBI+CY	10	26	3·4	16	12·7	–	Offspring (22)
7	M	SAA	MUD	TBI+CY	6	17	4·8	14	16·2	–	–
8	M	SAA	MUD	TBI+CY+ATG	17	31	5·1	9·7	17·8	–	Offspring (30)
9	M	SAA	MUD	TBI+CY+ATG	19	20	4·9	8	30·9	–	–
10	M	SAA	MRD	TBI+CY	16	21	7	13·5	25·1	–	Normal spermatogenesis (21)
11	M	RCC	MRD	TBI+CY	5	12	2·2	4·4	4·1	–	–
12	M	RCC	MUD	TBI+CY+ATG	14	19	3·6	6·4	19·4	–	–
13	M	RCC	MUD	TBI+CY+ATG	14	16	5·2	8·9	13·0	–	–
14	M	SAA	MUD	TBI+CY+ATG	9	19	NE	NE	NE	–	Offspring (19)
15	F	SAA	MRD	TBI+CY	6	22	21·6	8·7	–	1395·0	Menstruation (12)
16	F	SAA	MRD	TBI+CY	6	20	3·6	5·5	–	58·7	Menstruation (11)
17	F	SAA	MRD	TBI+CY+ATG	9	12	1·6	4·2	–	80·8	Menstruation (12)
18	F	SAA	MRD	TBI+CY	10	14	5·5	1·3	–	400·1	Menstruation (10)
19	F	RCC	MUD	TBI+CY+ATG	10	12	3·3	10·8	–	143·2	Menstruation (12)

BMT, bone marrow transplantation; LH, luteinizing hormone; FSH, follicle-stimulating hormone; M, male; F, female; SAA, severe aplastic anaemia; RCC, refractory cytopenia of childhood; MRD, matched related donor; MMRD, mismatched related donor; MUD, matched unrelated donor; TBI, total body irradiation; CY, cyclophosphamide; ATG, anti-thymocyte globulin; NE, not evaluated.
*The conditioning regimen consisted of 3 Gy of TBI and 200 mg/kg CY for MRD BMT. In the case of alternative donor BMT, 10 mg/kg ATG was added to the regimen.

men had fathered a child suggests the potential of the regimen to preserve fertility after SCT.

Fertility after SCT is generally likely to be preserved in younger patients (Loren *et al*, 2011; Sanders *et al*, 2011). All the five female patients in our study were pre-pubertal at the time of SCT; further studies including post-pubertal girls, young adults, or those who are older at SCT should be conducted. In male patients, because spermatogenesis is exquisitely sensitive to radiation (Howell & Shalet, 1998), semen analysis, which is not routinely performed, is necessary for definite evaluation of fertility.

The efficacy of the current conditioning regimen with CY and ATG has been confirmed for MRD BMT in SAA patients (Storb *et al*, 2001). Therefore, the 3 Gy TBI-containing regimens described in our study are no longer used for MRD BMT in SAA patients in our centre. In MUD BMT, favourable outcomes of 2 Gy TBI plus CY plus ATG regimen have been demonstrated in the National Marrow Donor Program study (Deeg *et al*, 2006). Therefore, it may be necessary to introduce a 2 Gy TBI regimen for MUD BMT in SAA patients in our centre. Further research is needed to determine which regimens are the least gonadotoxic but are equally effective for treatment; nevertheless, our results may serve as a guide on the gonadotoxicity of low-dose TBI and CY-containing regimens.

Authorship

JI and JO designed the study and wrote the manuscript; RF, YK, and MN collected the data and critically reviewed the manuscript; all authors approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interests.

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Keywords: gonadal function, low-dose total body irradiation, cyclophosphamide, severe aplastic anaemia, refractory cytopenia of childhood

First published online 21 June 2013
doi: 10.1111/bjh.12441

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■ *The Japanese Journal of Pediatric Hematology/Oncology* vol. 50(3): 374–377, 2013

【第54回日本小児血液・がん学会学術集会】シンポジウム10：長期フォローアップを取り巻くチーム医療

長期フォローアップ外来の現状

～理想と現実～

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要　旨

小児がんが治るようになって，長期フォローアップ（LTFU）の重要性が認識されてきた．LTFUにおける重要なポイントは，医療者・本人ともに原疾患と受けた治療によるリスクをきちんと認識しておくこと，LTFU外来に十分な時間を確保することである．他の専門外来との併診を要することも多く，連携に苦慮することも少なくない．晩期合併症で悩む人たちには，小児がん経験者のネットワークが心の支えになるケースも多く，その情報提供も有用である．今後ますます重要性が増すと考えられるLTFUがより円滑となるように，更なる取り組みが必要である．

キーワード：小児がん経験者，長期フォローアップ外来，晩期合併症，小児がん経験者の会

Key words: childhood cancer survivors, long term follow up clinic, late effects, Peer Support Group

I　はじめに

近年，小児がんの治療成績は著明に向上した．40年前までは，小児がん＝不治の病で，治ることがすべての目標であり，治った後の生活など考える由もなかった．治るようになった今，治療終了から数十年後に起こる可能性のある晩期合併症[1]や，本人の社会的な自立など，新たな課題に直面するようになった．

そして，小児がんの治癒後も，長期にフォローアップしていくことの重要性が認識されるようになった．しかしながら，このシステム作りは一朝一夕にできるものではなく，まだまだ十分とはいえないのが現状である．一方で，ようやくがん対策基本法に小児がんが盛り込まれた．今こそ長期フォローアップのシステムを充実させるときである．小児がんを乗り越えた人たちが，治癒後の長い人生を全うできるよう応援していくために，長期フォローアップ（以下，LTFU）外来がどのようにあるべきか考えた．

II　当科のLTFUの方法

1）血液腫瘍外来とは別枠で，長期フォローアップ外来を設定（週1回午前・午後）．
2）小児科外来にて（2012年度まで紙カルテだった当院の都合あり）．
3）小児科をベースに，小児科内の他専門外来・他科紹介・心理相談を行う．
4）完全予約制で，診療の時間をゆっくり確保し，受けた治療のリスクに応じて（FUガイドラインに応じて）行う[2,3]．
5）本人は，病気・治療の説明を受け，理解している．
6）他院で治療した人で，治療サマリー・紹介状持参での長期FUも行う．

当科では，以前より血液腫瘍外来の時間枠の中でLTFUを行っていた．しかしながら，血液腫瘍外来では，治療中や治療終了後間もない子ども達の診療に多くの時間を費やすので，LTFUに十分時間をとるために，別の曜日にLTFU外来枠を設けることにした．

身体的なことから社会的なことまでの幅広い悩みに応じられるように，完全予約制で（10歳から45歳）．受けた治療のリスクに応じて（FUガイドラインに応じて），定期的な経過観察を行っている．

理想的には，受診日に，小児がん経験者一人一人について，がん検診をも含む各科の診察・検査がプログラムされ，1日で完了するもの．縦と横の連携がしっかりとれていて，担当医が交代しても問題なく実施できるような長期フォローアップ外来が望ましいと考えるが，各科のマンパワー不足と，専門科・外来により診療日が制約されることから，なかなかそのようにうまく回らないのが現状である．

また，長期のフォローアップ期間中に構築してきた患者と医療者間の信頼関係は大きく，主治医の交代が円滑に進むかは，今後の課題である．

2013年7月3日受付，2013年7月3日受理
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長期フォローアップに入った時点で，本人は病気の説明を受けている．当科における本人への病名説明の基本方針は次のとおりである．

- ➢ 10歳以上（本人の理解度により7歳以上）は，できるだけ診断後早い時期に，本人へ病名と治療について話をする．
- ➢ 乳幼児期に発病した人へも，本人の理解の状況をみながら，10歳前後で病気の説明を行う．
- ➢ 治療終了後，再発の可能性が低くなった時期に，起こりうる可能性のある晩期合併症について話をする（がんの子どもを守る会のパンフレット[9]を利用）．（特に，喫煙の害については，喫煙を開始することの多い思春期・中学進級頃から繰り返し説明する）

長期フォローアップの必要性については，
- 小児がんは40年前まで不治の病だったこと．
- 乳幼児期に発症して，50歳以上になった人はほとんどいないので，ようやくいろいろなことがわかってきたこと．
- 病気自体・治療の内容・生活の環境・身体や細胞の老化など，いろんな要因で起こると考えられること．
- 予防できることを予防していくことが重要なこと．

を説明している．

III　課題；他の専門外来との併診について

当科の長期FU外来を行うときに，最も配慮を要するのが他の専門外来との併診である．その実態について調べてみた．

長期FU外来受診中の白血病患者（131人），固形腫瘍患者（115人）についてみてみると（図1），白血病では併診ありが35名（27%）に対し，固形腫瘍では43人（37%）であった．

併診の内訳をみると（図2），白血病では消化器内科，脳外科，婦人科，内分泌（小児科・内科），精神・神経科，整形外科，循環器内科の順で，固形腫瘍では，整形外科，内分泌（小児科・内科）循環器内科，婦人科，脳外科の順であった．固形腫瘍では，原疾患の治療の際に関わった科に加えて，晩期合併症の経過観察のために受診する科があり，白血病に比べて併診する科も多くなる．

消化器内科は，1990年以前の輸血によるC型肝炎や脂肪肝のフォロー，脳外科は白血病では二次性脳腫瘍関連のものが多い．婦人科は多くが移植後の性腺機能障害によるもので，内分泌は，成長ホルモン欠乏症や甲状腺機能低下症などである．整形外科は，白血病では骨壊死関連，固形腫瘍は多くが原疾患によるものである．治療終了後10年以上が経過して，腎機能や心機能に問題が出てくることもある．

併診科の診察日が，長期FU外来日と同じとは限らず，他科で治療や密な観察を要する場合は，そちらが主になっていき，気づくと小児科の受診が遠のいていることもある．また，就労者では時間と費用の問題も大きい．

IV　小児がん経験者が抱える内面的な問題

厳しい闘病生活を経て，一つ一つを自信として社会に出ている小児がん経験者はたくさんいるが，中には，抑うつ症状や心的外傷後ストレス障害を呈する人もいる．そのような場合には，臨床心理士によるカウンセリングを受けたり，精神科を紹介したりする．

闘病期間が長く，周囲から過剰に守られて育ち，同年齢

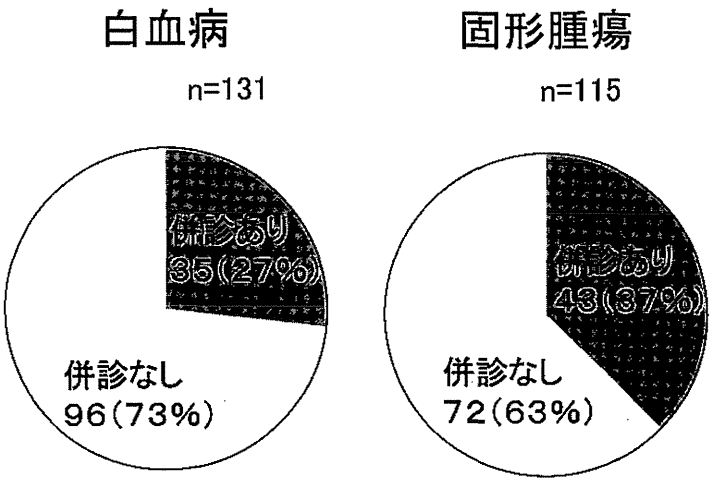

図1　他専門科との併診