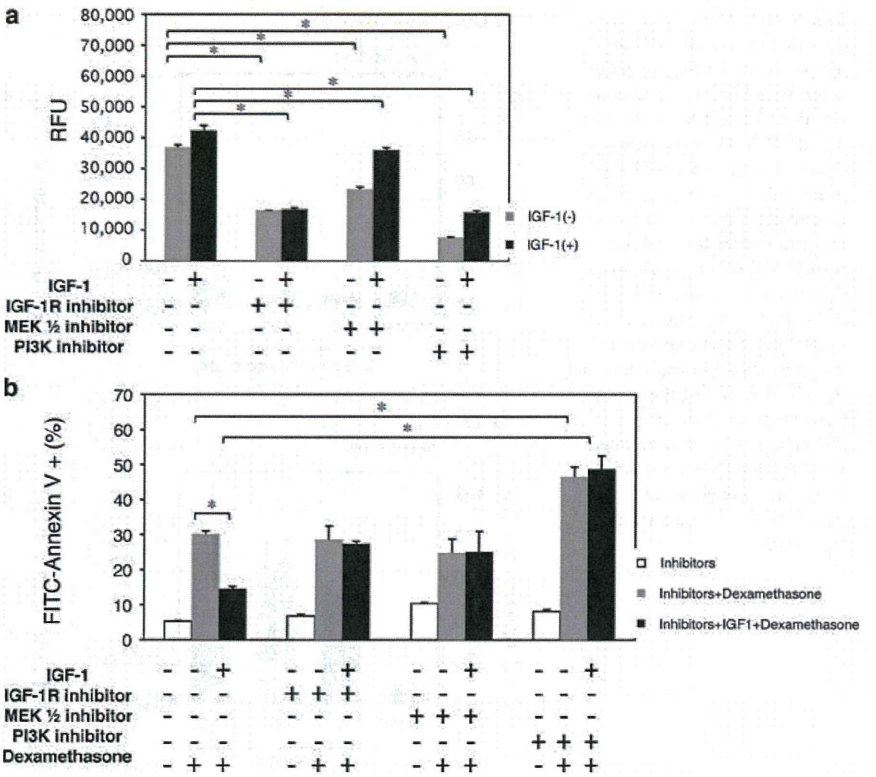

Fig. 7 Effect of inhibitors of insulin-like growth factor-1-related signaling molecules on the B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell line. RS4;11 cells were incubated with or without IGF-1R kinase inhibitor I-OMe-AG538 (50 μM), the mitogen-activated protein kinase extracellular signal-regulated kinase (MEK) 1/2 inhibitor U0126 (20 μM), phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (50 μM), and IGF-1 100 ng/mL for 60 h and resulting cell proliferation (**a**) and the frequencies of apoptotic cells (**b**) were examined by ATP assay and Annexin V-binding assay, respectively. Each experiment was performed in triplicate, and the mean ± SD of the data are shown. Data are representative of three independent experiments. *significant ($p < 0.01$)

Fig. 5b, when IGF-BPs were added simultaneously, the enhancement of phosphorylation of each protein was inhibited.

Effect of IGF-1 on apoptosis induction of BCP-ALL cell lines

We then examined whether IGF-1-stimulation could inhibit apoptosis induction in BCP-ALL cells. As shown in Fig. 6, VP-16 and irradiation induced apoptosis in both NALM-16 and RS4;11 cells, whereas DEX-treatment induced apoptosis in RS4;11 cells, but not NALM-16 cells. When IGF-1 was added to the culture, apoptosis induced by DEX was partially inhibited, whereas apoptosis mediated by other treatments was not affected.

Next, we examined the effect of inhibitors for kinases. As mentioned above, stimulation of IGF-1R by the binding of IGF-1 is thought to be mainly mediated by MAPK and PI3K pathways. Although incubation with inhibitors for IGF-1R, MAPK, and Akt reduced cell proliferation, all of them induced apoptosis. When cells were incubated with a combination of IGF-1R inhibitor and IGF-1, the proliferative effect of IGF-1 was completely blocked, whereas, when cells were incubated with either MEK or Akt inhibitor, the proliferative effect of IGF-1 was only partially blocked. On the other hand, the inhibition of DEX-induced apoptosis mediated by IGF-1 was completely blocked by IGF-1R inhibitor (Fig. 7b). In addition, either MEK or Akt inhibitor also completely blocked the inhibition of DEX-induced apoptosis mediated by IGF-1.

Discussion

The results of the present study clearly showed the expression of IGF-1R by BCP-ALL cells. As we presented, most BCP-ALL cell lines expressed IGF-1R, whereas IGF-1R expression was limited in some cases of clinical samples. Although the precise reason for the inconsistency in the frequency of IGF-1R expression between cell lines and fresh ALL cells is unknown, it may be that IGF-1R expression is an advantage for cell growth and, thus, IGF-1R-positive cases tend to more frequently be able to be established as a cell line. Indeed, IGF-1 introduces BCP-ALL cells in the S phase of the cell cycle and promotes cell proliferation in a dose-dependent manner.

On the other hand, we showed that IGFBP-1, -3, and -4 inhibit IGF-1-mediated cell proliferation. Our data indicated that the presence of these IGFBPs inhibited intracellular signaling mediated by IGF-1. Therefore, it is most likely that these IGFBPs obstruct the binding of IGF-1 to IGF-1R and, thus, inhibit IGF-1 stimulation. However, a number of studies reported the possibility of a direct effect

of IGFBPs independent of IGF-1 function [16]. Further experiments need to be performed to clarify the precise mechanism of the effect of IGFBPs on IGF-1-mediated proliferation of BCP-ALL cells.

We also showed that IGF-1 partially inhibits apoptosis induced by DEX, whereas it did not affect apoptosis mediated by other causes, including VP-16 and irradiation. Our data indicate that the mechanism of apoptosis induction mediated by DEX and others might be different and IGF-1 stimulation can only overcome DEX-mediated apoptosis among the three causes of apoptosis. Since corticosteroid is a common drug for the treatment of ALL, it is possible that the expression of IGF-1R may affect the therapeutic response in BCP-ALL cells. Although previous studies reported no significant correlation between either gene expression of IGF system components in ALL cells or the serum IGF-1 level and prognosis [14, 21], more precise studies should be performed to evaluate the prognostic significance of IGF-1R expression in BCP-ALL cells.

Interestingly, both MEK inhibitor and Akt inhibitor completely blocked the inhibition of DEX-induced apoptosis mediated by IGF-1, while the proliferative effect of IGF-1 was only partially blocked by each inhibitor. Our data suggest that activation of both the MAPK and Akt pathways is required for the inhibition of DEX-induced apoptosis, whereas the activation of one of these pathways can support the proliferation of BCP-ALL cells, at least in part.

In conclusion, we observed IGF-1R expression in both clinical samples and cell lines of BCP-ALL cells that mediate cell proliferation and the anti-apoptotic effect against DEX. Although more detailed experiments are clearly needed, our findings indicate the possible involvement of the IGF-1 system in the proliferation and survival of some BCP-ALL cases, and may provide a model for investigating the molecular basis of the biological effect of IGF-1 on BCP-ALL cells. Since fully human antibody directed against IGF-1R is now clinically available [22], a new therapeutic strategy might be identified.

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Conflict of interest We have no financial relationships or conflicts of interest related to this manuscript to declare.

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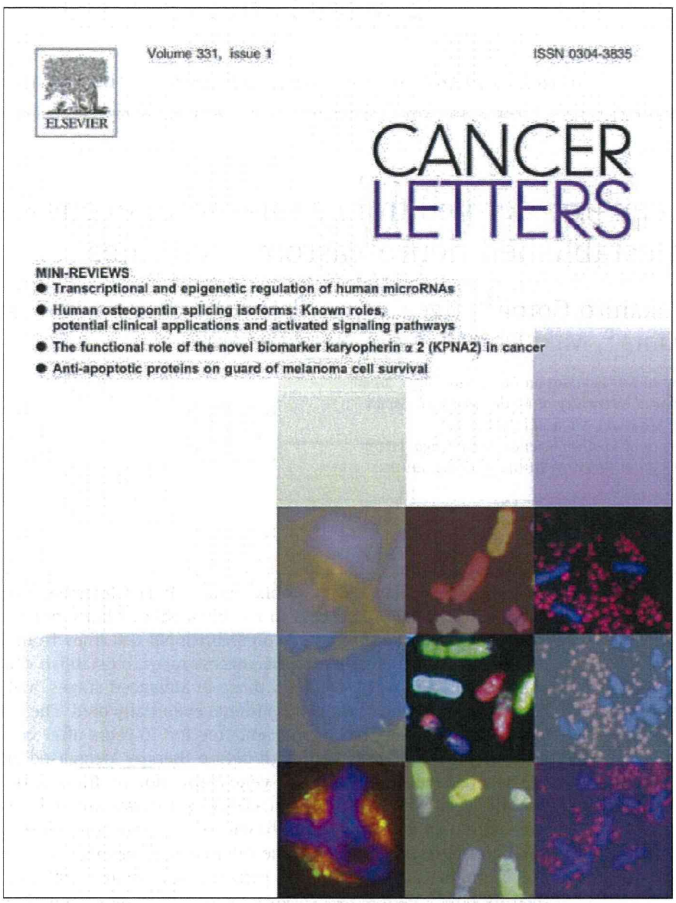

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A *MYCN*-amplified cell line derived from a long-term event-free survivor among our sixteen established neuroblastoma cell lines

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ABSTRACT

Although more than 110 neuroblastoma (NB) cell lines have been established, there have been neither reports on the rate of success to establish NB cell lines, nor well-documented NB cell lines from long-term-survivors. We attempted to establish NB cell lines from 114 patients. Sixteen NB cell lines were established from 12 patients. The success rates to establish cell lines were 1.4% (1/70) from patients in early stages, 25.0% (11/44) from those in advanced stages, and 10.5% (12/114) from those in all stages respectively. Eleven of these 12 patients eventually died. The surviving patient, who was in stage 4 with *MYCN*-amplification, has been event-free for 19 years after completing therapy. The serum *MYCN* DNA level in patient TK was very high before therapy, decreased after chemotherapy, and has remained at the normal levels until now. The gene expression profiling of the primary tumor and the K-N-TK cell line was analyzed with an NB-specific cDNA microarray, and indicated that the probability of 5-year survival was extremely low. Microarray-based comparative genomic hybridization (CGH) analysis indicated that genomic aberration profiles of the cell line were uncommon, with *MYCN* amplification, 17q gain and 11q loss. A unique KP-N-TK cell line, established from an event-free survivor, will be a useful tool for investigating how a patient can survive a tumor with an extremely poor prognosis.

1. Introduction

Neuroblastoma (NB) is a common type of malignant solid tumor in childhood, arising from neural crest precursors. The first NB cell lines, NB-1 and SK-N-SH, were established in 1973 [1,2]. Since then, more than 110 NB cell lines have been reported [3]. Most NB cell lines were generated from tumor samples obtained from patients with an advanced stage of neoplastic disease.

NB cells to grow progressively in a tissue culture flask enable us to establish a permanent cell line, and the inverse correlation with the survival rate of patients, from whom the NB cell lines were derived, was reported [3,4].

This study was undertaken to estimate the success rate to establish NB cell lines from 114 patients with early or advanced stages, to know the patient's outcomes whose cell lines were generated. For the KP-N-TK cell line, established from a long-term survivor of patient TK, we assayed *MYCN* DNA levels of primary tumor cells, the cell line and serum levels before and after therapy to monitor the residual tumor cells during the patient's clinical course. Finally, microarray analyses of both gene expression profiles and genomic DNA aberrations in primary tumor cells of patient TK and/or the KP-N-TK cell line were analyzed to see if they could explain patient TK's long-term survival.

2. Materials and methods

2.1. Patients from whom we attempted to establish NB cell lines

The patients at the Hospital of Kyoto Prefectural University of Medicine, University of Miyazaki, and the affiliated hospitals in Japan, were enrolled in this study with the informed consent of their parents. The study was conducted under research protocols approved by the institutional review boards. From 1983 to 2005 we attempted to establish NB cell lines from 114 patients (37 patients with stage 1 NB, 26 stage 2, 9 stage 3, 35 stage 4, and 7 stage 4S). NB was detected in 67 of these patients by the Japanese Mass Screening System (Table 1).

2.2. Clinical outcome of 114 patients and clinical history of patient TK

Five-year overall-survivals (OSs) were high in the early stages (100% in stage 1, 96.1% in stage 2 and 85.7% in stage 4S respectively) and low in advanced stages (55.5% in stage 3 and 25.7% in stage 4 respectively) (Table 1). Among the 114

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attempts to establish an NB cell line, only one was successful. The source of the cell line was a long-term survivor of NB, a Japanese male referred to as patient TK. In February 1992, at age 13 months, he presented with anemia and right-exophthalmos. Clinical examination revealed a left upper abdominal mass, and magnetic resonance imaging showed a primary abdominal tumor arising from the left adrenal gland and a right orbital bone metastasis. Morphological and surface membrane analysis [5] of bone marrow aspirates revealed that most (80%) of the normal hematopoietic progenitor cells had been replaced by tumor. The right orbital bone and bone marrow metastases led to NB with stage 4. High levels of the catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), were identified in the patient's urine.

Biopsy of the primary tumor prior to chemotherapy showed small-to medium-sized round tumor cells with poor stromal development and a high mitosis–karyorrhexis index (MKI) (437/5000) (Fig. 1). The tumor was diagnosed as an unfavorable type of NB (Schwannian stroma-poor, undifferentiated subtype with high MKI) according to the International NB Pathology Classification [6].

The patient was treated with six courses of chemotherapy consisting of a combination of cyclophosphamide, etoposide, doxorubicin and cisplatinum, followed by a delayed-primary operation. Complete remission of primary and metastatic lesions was confirmed by magnetic resonance imaging, MIBG scintigraphy, bone marrow aspiration and urinary VMA and HVA levels. He was then given megatherapy with peripheral blood stem cell transplantation (PBSCT) [7,8]. Treatment was terminated in April 1993, and the patient has been event-free for 19 years after completing therapy.

2.3. Cell culture to establish NB cell lines

Tumor samples from the 114 patients (Table 1) for cell culture were obtained from biopsy, operation or autopsy and finely minced with scalpels and cultured. For patient TK, prior to chemotherapy, primary tumor cells but not bone marrow tumor cells were obtained by biopsy in March 1992 and cultured. Mononuclear cell fractions of cells from bone marrow metastatic samples were prepared by Ficoll-Hypaque centrifugation. Cells were cultured in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 15% heated-inactivated fetal calf serum at 37 °C in 5% CO_2 in air. The medium was exchanged every 3–4 days [5]. Cell lines were considered to be established when they have been maintained for more than 60 passages over a 2-year period.

2.4. Identification of KP-N-TK cell line from patient TK

A Cell ID System (Promega, Madison, WI) based on short tandem repeats (STR) was used to compare KP-N-TK cells with WBC and cells from the primary tumor of patient TK [9]. Genomic DNA was used for PCR analyses. The tool of online verification of cell line is available from DSMZ Online STR Analysis (http://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html). The identity of two cell lines (A and B) is expressed as an evaluation value (EV) calculated as EV = {Number of coincident peaks of STR profiles between A and B} × 2/{Total number of peaks of STR profiles in A and B}. EV values greater than 0.9 indicate that the two cell types are derived from the same origin.

2.5. Assay of tumor and serum MNA by real-time quantitative PCR

MYCN amplification (MNA) in the original tumor sample and KP-N-TK cell line was determined by our developed real-time quantitative PCR [10] instead of the conventional Southern blot analysis [11,12]. DNA was prepared from tumor tissue, cell line and stored serum (200 μL). The *MYCN* (2p24) copy number of a sample of DNA was determined by the ratio of the *MYCN* dosage to the *N-acetylglucosamine kinase gene* (*NAGK*) (2p12) dosage (*M/N* ratio). This method has several advantages over FISH and Southern blotting methods, including short turnaround time (4 h), much less effort, well-correlated *M/N* ratio between tumor DNA and serum DNA, and more highly accurate detection of MNA gene status. Another clinical benefit of the serum *M/N* assay is that the *M/N* ratio can be used as a tumor marker [10].

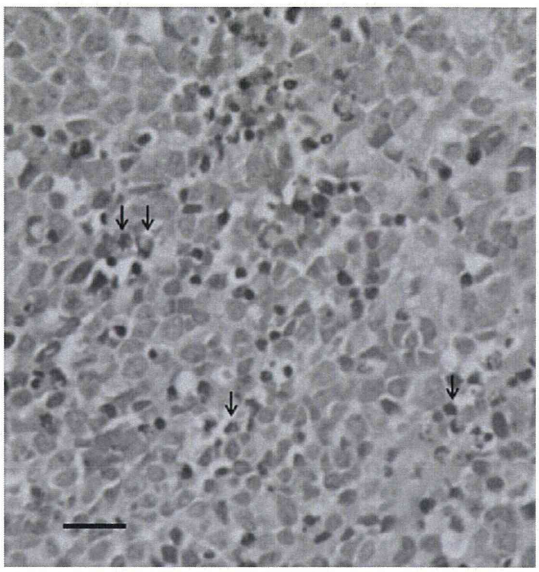

Fig. 1. Histological appearance of the primary tumor of patient TK on biopsy. Schwannian stroma-poor, undifferentiated medium to large size tumor cells were seen. The mitosis–karyorrhexis index (MKI), indicated by arrows, was very high (437/5,000). Scale bar: 20 μm.

2.6. Gene expression profiling of the tumor by the NB-proper cDNA microarray

We previously constructed an NB diagnosis mini-chip which harbors the 200 top-ranked prognosis-related genes and developed a computational algorithm for prognosis prediction by using the 50 gene profiles in the tumor (Table 1S).

The survival probability of each patient at 5 years after diagnosis was calculated by the computational algorithm as a posterior value (ranging from 0 to 1) [13,14].

2.7. Microarray-based comparative genomic hybridization of KP-N-TK cell line

DNA copy number alterations in the tumor were determined with an array-based CGH using a DNA chip carrying 2464 BAC clones (Agilent Technology, Santa Clara, CA) as previously reported [14,15]. The KP-N-TK cell line (60 passages, January 1995) was used for this assay because the amount of primary tumor cells was insufficient.

3. Results

3.1. Establishment of NB cell lines

NB cell lines were identified as NBs from the clinical features of the patients, surface-membrane analysis with a panel of nine monoclonal antibodies [5], and by a cytoskeletal protein analysis with anti-neurofilament antibody [16,17], as previously reported.

From 114 patients with NB, 16 NB cell lines, derived from 12 patients, were established.

The success rate of establishing cell lines was 0% (0/37 patients) in stage 1, 0% (0/26) in stage 2, 11.1% (1/9) in stage 3, 28.6% (10/35)

Table 1
Effect of NB stage on the 5-year over-all survival, success rate of establishing NB cell lines from 114 patients.

Stage	Primary culture	Establishment of cell line	
	Survival pt[a]/total pt (5-year OS[b] %)	Success rate (%)	Survival pt/pt established cell line (5-year OS %)
Stage 1	37/37 (100)	0/37 (0)	0/0 (0)
Stage 2	25/26 (96.1)	0/26 (0)	0/0 (0)
Stage 3	5/9 (55.5)	1/9 (11.1)	0/1 (0)
Stage 4	9/35 (25.7)	10/35 (28.6)	1/10 (10)
Stage 4S	6/7 (85.7)	1/7 (14.3)	0/1 (0)
Total	82/114 (71.9)	12/114 (10.5)	1/12 (8.3)

The success rate of establishing cell lines from early (1, 2 and 4S) stage was 1.4% (1patient/70 patients), and from advanced (3 and 4) stage was 25.0% (11/44).

[a] Patient.

[b] Overall-survival.

in stage 4, 14.3% (1/7) in stage 4S and 10.5% (12/114) in all stages, respectively (Table 1). Five-year OS rate (%) among 12 patients, from whose cell lines were established, was 0% (0/1 patient) in stage 3, 10% (1/10) in stage 4, 0% (0/1) in stage 4S and 8.3% (1/12) in all stages (Table 1).

Table 2 lists the characteristics of 16 NB cell lines from 12 patients in our laboratories. Ten of the samples (62.5%) used to establish these cell lines were collected before any therapy, four samples (25.0%) were collected during chemotherapy, and two samples (12.5%) were collected at autopsy. Eleven cell lines were from NBs with MNA and 5 from NBs without MNA. Of these 12 patients, 11 patients eventually died and only one patient (patient No. 12) survived (Table 2) [5,11,12,17–28].

3.2. Establishment of the KP-N-TK NB cell line

Minced tumor cells obtained from a biopsy in March 1992 (before therapy) grew in the form of adherent cells. The cells were spindle-shaped or focally aggregated with neurite processes, and were maintained for more than 60 passages. The cell line, designated as KP-N-TK, was established in January 1995.

3.3. Confirmation that the KP-N-TK ell line was derived from patient TK

STR analysis of primary TK tumor and the KP-N-TK cell line was identical. STR analysis yielded an EV between both primary TK tumor (sampled March 1992) and the KP-N-TK cell line (July 1999), and WBC (November 2009) from patient TK of 0.966, indicating that the three cell types had the same origin. STR analysis of the primary TK tumor and the KP-N-TK cell line revealed a loss of heterozygosity (LOH) at D16S539 (16q24-qter) and Amelogenin X (Xp22.1–22.3, Y) (Table 3). The LOH results were in agreement with those of CGH described below, revealing whole losses of 16q and X (data not shown).

Table 2
Characteristics of 16 NB cell lines established from 12 patients by our laboratories.

Cell line	Patient number[a]	Age y m[b]	Primary tumor	Stage	Metastasis	Sample	Therapy	*MYCN* amplification[c]	Outcome	Refs.
KP-N-RT-BM	01	1 y 2 m	Adr gl[d]	4	BM[e], Bone	BM	Before[f]	50	Dead	[5,12,17–19]
KP-N-RT-LN	01	1 y 2 m	Adr gl	4	BM, Bone, LN[g]	LN	Before	50	Dead	[5,11,20,21]
KP-N-RT-BMV	01	1 y 2 m	Adr gl	4	BM, Bone	BM	Before	100	Dead	[12]
KP-N-SILA	02	5 y	Adr gl	4S	LN, Bone	LN	Autopsy[h]	1	Dead	[16,17,22]
KP-N-SIFA	02	5 y	Adr gl	4S	LN, Bone	Bone	Autopsy	1	Dead	[17–19,23,24]
KP-N-YN	03	2 y	Adr gl	3	LN	Delayed primary[i]	During[j]	100	Dead	[16,17]
KP-N-AY	04	2 y 6 m	Adr gl	4	LN, BM	BM	Before	50	Dead	[25]
KP-N-AYR	04	2 y 6 m	Adr gl	4	LN, BM	BM	During	50	Dead	[25]
MP-N-MS	05	1 y 6 m	Adr gl	4	BM	BM	Before	50	Dead	[17]
KP-N-YS	06	4 y	Adr gl	4	BM	BM	Before	10	Dead	[17]
MP-N-TS	07	2 y 8 m	Adr gl	4	Bone, Gingiva	Primary[k]	Before	1	Dead	[26–28]
KP-N-HN	08	4 y	Adr gl	4	BM	BM	During	1	Dead	[26]
KP-N-NY	09	5 y	Adr gl	4	BM	Delayed primary	During	1	Dead	[26]
KP-N-SK	10	2 y 2 m	Adr gl	4	Bone, BM	BM	Before	45	Dead	Unpublished
KP-N-YuNo	11	1 y 5 m	Retro[l]	4	LN	Primary	Before	10	Dead	Unpublished
KP-N-TK	12	1 y 1 m	Adr gl	4	BM, Bone	Primary	Before	20	Alive	[23,24]

Sixteen NB cell lines were established from 12 patients. KP-N-TK cell line is the only cell line whose patient had a long-term (19-year) event-free survival.
[a] Patient number: A same number indicates the established cell line from the same patient.
[b] y m: year month.
[c] MYCN amplicication: MYCN copy number by Southern blot.
[d] Adr gland: adrenal gland.
[e] BM: bone marrow.
[f] Before: Sample obtained before any therapy.
[g] LN: lymph node.
[h] Autopsy: Sample obtained at autopsy on tumor death.
[i] Delayed primary: delayed primary tumor.
[j] During: Sample obtained during therapy.
[k] Primary: primary tumor.
[l] Retro: retroperitoneal.

Table 3
STR analysis of primary TK tumor, KP-N-TK cell line and WBC from patient TK.

STR	Locus	Primary TK tumor		KP-N-TK cell line		WBC from patient TK	
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
TPOX	2p23–2pter	8	11	8	11	8	11
D5S818	5q23.3–32	8	12	8	12	8	12
CSF1PO	5q33.3–34	11	12	11	12	11	12
D7S820	7q11.21–22	11	–	11	–	11	–
TH01	11p15.5	7	9	7	9	7	9
vWA	12p12-pter	17	–	17	–	17	–
D13S317	13q22-q31	9	11.2	9	11.2	9	11.2
D16S539	16q24-qter	11	–	11	–	9	11
D21S11	21q11–21q21	29	–	29	–	29	–
Amelogenin X	Xp22.1–22.3 and Y	X	–	X	–	X	Y

STR analysis of primary TK tumor, KP-N-TK cell line and WBC from patient TK indicated that the three cell types had the same origin.

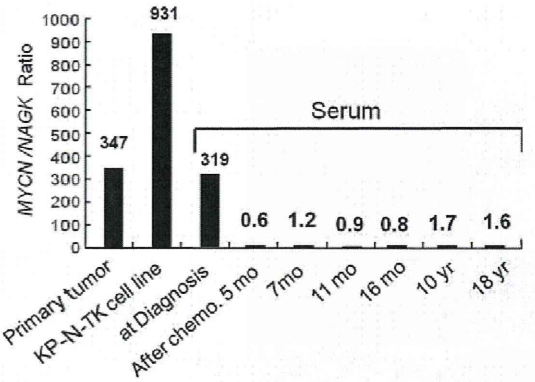

Fig. 2. *MYCN* DNA levels of primary TK tumor, KP-N-TK cell line and serum before and after chemotherapy by quantitative PCR.

3.4. MYCN DNA levels of primary tumor, cell line and serum

The *MYCN* DNA levels, expressed as *M/N* ratios, in the primary tumor and serum at diagnosis were very similar (347 and 319, respectively). The *M/N* ratio in KP-N-TK cells at passage 70 (931) was 2.7 times higher level than that of the primary tumor (347), suggesting the selection of *MYCN*-amplified clone during the culture period. In patient TK, the serum *MYNC* level was 319 at diagnosis in March 1992, decreased to 0.6 in September 1993 after 5 months of chemotherapy, and has consistently remained low (below 2.2) for the last 18 years (Fig. 2).

3.5. Gene expression signatures of primary TK tumor and the KP-N-TK cell line

The gene expression profiles of primary tumor tissue obtained in March 1992 and KP-N-TK cells from passage 62 were determined with a microarray spotted with the 200 top-ranked genes for clinical use (Table 1S) [13,14]. Four of nine genes associated with a poor prognosis were strongly expressed, and five of seven genes associated with a favorable prognosis were weakly expressed in both the primary TK tumor and KP-N-TK cells. The unfavorable prognosis genes were ribosomal protein genes *RPL18A* (Table 1S, Gene No. 2) and *RPLP0* (Gene No. 24), translation initiation gene *EEF1G* (Gene Nos. 3 and 22) and metabolism gene *enolase 1* (Gene No. 10). The favorable prognosis genes were neuronal differentiation genes *tubulin α* (Gene No. 46), *peripherin* (Gene Nos. 38 and 50), *neuromodulin* {*GAP43*}(Gene No. 44), and *HMP19* (Gene No. 42) and catecholamine metabolism gene *tyrosine hydroxylase* {*TH*} (Gene No. 28) (Fig. 3).

With regard to the *MYCN* expression we have a set of expression data obtained from 50 reference samples (41 non-*MYCN* amplified and 9 *MYCN*-amplified) by the microarray carrying the 200 top-ranked genes for clinical use (Fig. 3) [13]. Average log2 expression ratios in tumors with and without MNA were 1.243 and -0.015, respectively. In contrast, those of primary TK tumor and the KP-N-TK cell line were 0.312 and 1.046, respectively (average value of Gene No. 7 and Gene No. 9 in Fig. 3). The expression levels of *MYCN* of the primary TK tumor and the KP-N-TK cell line were less than those in typical *MYCN*-amplified tumors, and the expression levels in the cell line were higher than those in primary tumor cells.

The expression level of *NTRK1* (*TRKA*) expression (Gene No. 47) was moderate in both samples (Fig. 3).

The posterior values of primary TK tumor and the KP-N-TK cell line at 5 years after diagnosis were 0.003 and 0.001, respectively, showing that the prognosis of patient TK, based on the mini-chip algorithm, was extremely poor [13,14] .

3.6. Genomic aberration signature of KP-N-TK cell line

The array CGH analysis indicated that the KP-N-TK cell line had multiple chromosomal aberrations (data not shown). Based on four of these characteristics (chromosome 1 without deletion of 1p36, chromosome 2 with MNA at 2p24, chromosome 11 with deletion of 11q23 and chromosome 17 with 17q21-qter gain), the KP-N-TK cell line was classified as a GGP3a tumor which is an uncommon feature among NB tumors [14,15].

4. Discussion

NB cell lines are some of the first and most widely used human tumor cell lines. More than 110 established NB cell lines have been reported in a review [3]. Our cell lines, like most other NB cell lines [3,4], were established from patients with advanced stage diseases. Our established cell lines have been used for many studies, including studies of neuronal or schwannian cell differentiation [11,12,18–21], cell surface membrane analysis by monoclonal antibodies [5], S-type cells expressing smooth-muscle-cell phenotypes [16,17], drug resistance [25], signal transduction of neurotrophic-factors [18,26,27], signal transduction on apoptosis [23,24], and molecular biology of oncogenes including MNA [11,12,28] (Table 2).

An accurate success rate for establishing NB cell lines from primary cell cultures by using the identical procedure like in our laboratories has never been reported. Our success rate to establish cell lines from early (1, 2 and 4S) stage was 1.4% (1/70 patients). That is, the KP-N-SILA and KP-N-SIFA cell lines [16–19,22–24] were established from only one patient SI with favorable stage 4S among 67 patients detected by the Japanese Mass Screening System. In contrast, the success rates of cells from advanced (3 and 4) stage was 25.0% (11/44 patients), which were higher than expected (Table 1). The prognosis of patients whose NB cell lines are established is reported to be extremely poor [3,4]. Although a few personal communications are available, no well-documented NB cell lines with long-term survivors have been reported. Among our 12 patients whose cell lines were established, only patient TK survived.

Recently the International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification by the statistically significant and clinically relevant factors [29]. Of those the 5-year event-free survival (EFS) rate of patient TK by nine potential prognostic factors were: 35% based on his INSS stage (stage 4), 49% based on his age (over 365 days), 40% based on histological classification (unfavorable), 63% based on grade of NB differentiation (undifferentiated), 37% based on high MKI, 26% based on MNA status, 55% based on ploidy (diploid), 35% based on 11q status (aberration), and 41% based on 17q gain respectively. All of these factors except for tumor differentiation and ploidy had predicted 5-year EFSs of less than 50%, putting patient TK in a high risk group.

MNA is strongly associated with rapid tumor progression, which makes MNA the most powerful prognostic factor. The inverse relation between number of *MYCN*-gene copies and progression-free survival was significant among patients with stage 4 [30].

We previously reported a quantitative PCR method for measuring circulatory and tumor *MYCN* copy number [10]. This method has several advantages over FISH and Southern blotting methods as described in Materials and Methods. The higher *MYCN* status in primary tumor obtained by quantitative PCR (Fig. 2; *M/N* ratio = 347) is more accurate than the *MYCN* status obtained by Southern blotting (21 copies as compared with the single copy number control tissue of human placental DNA) (data not shown) [11,12]. It also shows a poorer prognosis for patient TK than the *MYCN* status estimated by Southern blotting.

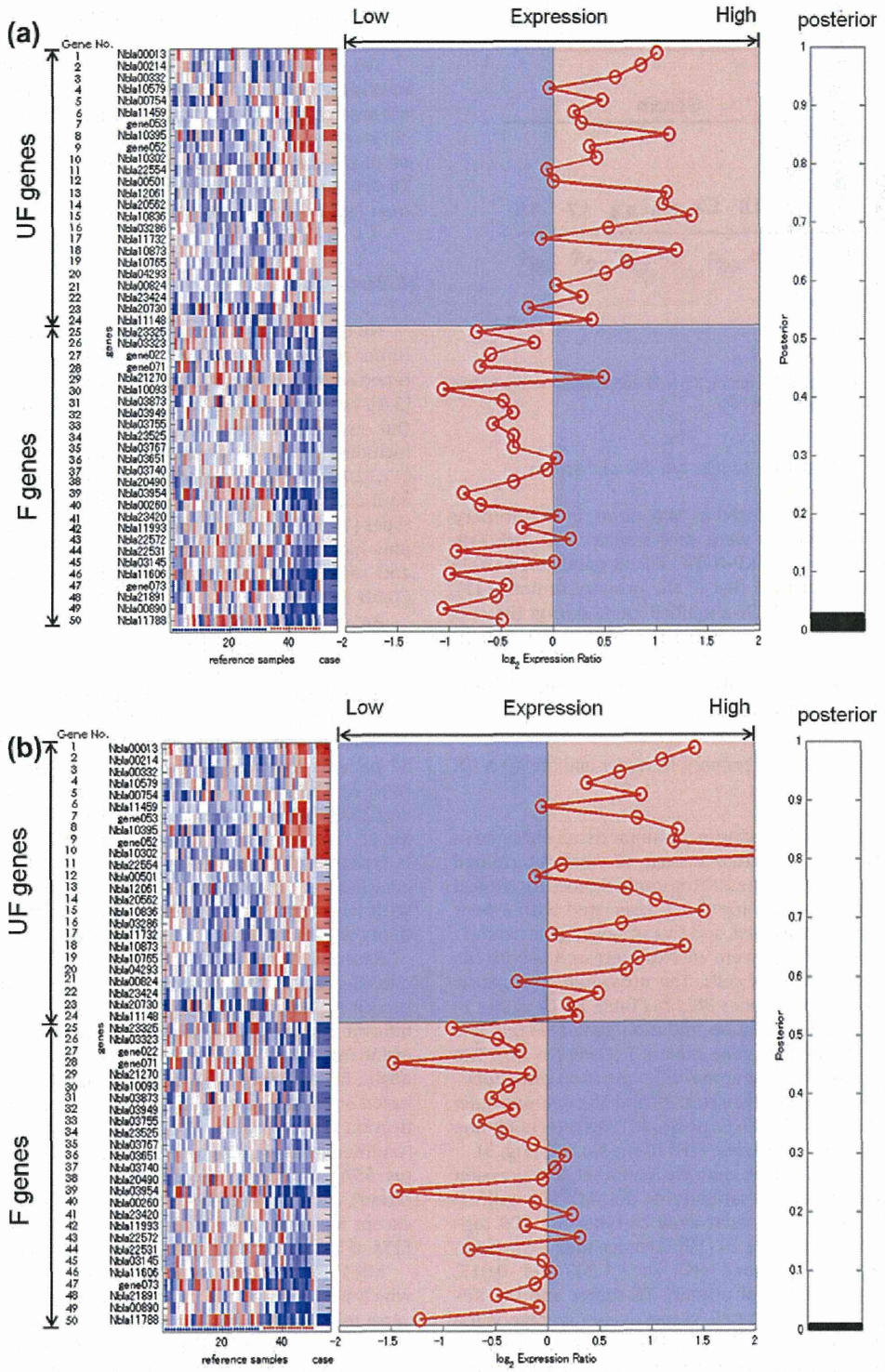

Fig. 3. Gene expression signatures of selected 50 genes in primary TK tumor and KP-N-TK cell line for predicting NB prognosis. (**a**) Primary TK tumor and (**b**) KP-N-TK cell line Twenty-four genes related to unfavorable prognosis (UF genes) and 26 genes related to favorable prognosis (F genes), listed in Table 1S, were analyzed. From left to right panels, gene number ($n = 50$), gene ID, gene expression profile of 50 reference samples, gene expression profile of primary TK tumor or KP-N-TK cell line, and posterior value are shown. The blue and red colors in the expression matrix show the high and low expression, respectively.

The clinical benefit of the serum *M*/*N* assay is that the *M*/*N* ratio can be used as a tumor marker [10]. The prognosis of patient TK was predicted to be very poor based on the cell line success and the INRG classification as described above. The *MYCN* copy number in human NB is usually consistent within a tumor, not only at different tumor sites, but also at different times in clinical courses

[31]. Therefore, serial serum levels of *MYCN* DNA in patient TK consequently demonstrated that the eradication of residual NB cells and the chemosensitive TK tumor cells by the chemotherapy with PBSCT. Thus, this is the first report of long-term (18-year) monitoring of the serum *M*/*N* ratio as a potent tumor maker before and after chemotherapy (Fig. 2).

In the primary tumor of patient TK and the KP-N-TK cell line, unfavorable prognosis genes (related to protein synthesis, and metabolism) were strongly expressed, whereas favorable prognosis genes (related to neuronal differentiation and catecholamine metabolism) were lowly expressed.

In the reference set of 50 tumors, 3 of 9 tumors with MNA and one of 41 tumors without MNA had lower and higher expression ratios of *MYCN* mRNA, respectively (Fig. 3), as compared to the primary TK tumor. The former three patients with MNA but lower expression of *MYCN* mRNA died of cancer within 2 years after diagnosis. Our previous expression profiling of 136 NB clearly showed that higher *MYCN* mRNA expression is one of the top-ranked poor prognosis-related genes as well as MNA [13], however, we do not have enough evidence so far, due to the small number of such cases, to conclude that low *MYCN* mRNA expression, despite of MNA, could explain long-term survivors.

The expression profiles of the TK tumor and the KP-N-TK cell line were similar, suggesting that the cell line had maintained the gene expression profile of the tumor. Posterior value is the probability of survival at 5-years after diagnosis calculated from these two gene expression profiles, predicting the possibility of survival of patient TK was nearly 0% (Fig. 3) [13,14].

Our CGH studies indicated that the KP-N-TK cells had MNA, 11q loss and 17q gain, but not 1p loss, and were distinguished from other NBs and were classified into uncommon subgroup of GGP3a, among GGP tumor groups ($n = 77$) with partial chromosomal gains/losses. Among 236 samples examined, only one death patient had GGP3a tumor, so it was difficult to define the 5-year OS rate for this genomic group. The 5-year OS rate of 15 patients with GGP3s tumor, with single copy of *MYCN*, 11q loss and 17q gain, was 59%. On the other hand, GGP1a ($n = 22$), with MNA, 1p loss and 17q gain showed 44% of 5-year OS rate. Since the effect of 1p loss and 11q loss in the NB survival seems to be similar, GGP3a might be similar survival rate to those of GGP1a or GGP3s, although the sample number in GGP3a was very small [14,15].

The unexpected survival of patient TK might be caused by the very unfavorable gene expression (Fig. 3) and uncommon genomic aberration (MNA, 11q loss and 17q gain) of TK tumors and/or the KP-N-TK cell line which distinguished them from the other major *MYCN*-amplified tumors. Some specific biological and genetic characteristics of TK tumor cells related to growth, metastasis, survival, differentiation, apoptosis, chemosensitivity to anti-cancer drugs or host immune response may have affected on the prognosis of patient TK. New generation technologies such as whole genome SNP typing and sequencing, micro-RNA profiles and epigenetic modifications will help to understand the mechanisms involved in overcoming the poor prognosis of patients with *MYCN* amplified tumors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.011.

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