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Characterization and chemosensitivity of two human malignant peripheral nerve sheath tumour cell lines derived from a patient with neurofibromatosis type 1

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Abstract Two new cell lines, designated NMS-2 and NMS-2PC, were established in vitro from a malignant peripheral nerve sheath tumour (MPNST) in the right thigh and a retroperitoneal lesion of a 30-year-old man with neurofibromatosis type 1 (NF1). The NMS-2 cell line was derived from the first tumour, and the NMS-2PC cell line from a retroperitoneal metastatic tumour detected 9 months later. Cultured NMS-2 cells showed epithelioid features, while NMS-2PC cells showed fibroblast-like features. However, both cell lines were strongly positive for S-100 protein. The transplanted NMS-2 and NMS-2PC tumours showed the same histological features typical of MPNST. Chromosomal analysis revealed that only the NMS-2 cells had a t (1;2) chromosomal translocation. Chemosensitivity tests demonstrated that NMS-2PC cells were far more sensitive than NMS-2 cells to Adriamycin and etoposide, which had been used clinically. All-*trans*-retinoic acid induced a morphological change in NMS-2PC cells so that they were no longer fibroblast-like, but epithelioid cells. We believe the epitheloid components in the MPNST were derived from typical spindle cells.

Key words Malignant peripheral nerve sheath tumour · Cultured cell line · Neurofibromatosis type 1 · Chemosensitivity

Introduction

Malignant peripheral nerve sheath tumour (MPNST), which accounts for about 10% of all soft tissue sarcomas, is considered to be a spindle cell sarcoma arising from any cell in the nerve sheath, including Schwann cells, perineural fibroblasts, or fibroblasts [4, 7, 26]. MPNST occasionally exhibits considerable histological variation within a tumour. Approximately half of all cases occur in patients with neurofibromatosis type 1 (NF1), and despite aggressive surgery and adjuvant chemotherapy the tumour tends to recur locally and to spread widely, especially to the lungs. The prognosis remains poor [8, 14, 25].

There are six cultured cell lines derived from human MPNST [5, 10, 11, 21, 23, 24]. Four of these were derived from tumours that developed in patients with NF1. We have successfully established two new MPNST cell lines, designated NMS-2 and NMS-2PC, which were derived from a patient with NF1. These cell lines have been characterized with respect to morphology, growth properties, immunocytochemical properties, karyotype and chemosensitivity. In this paper, we clarify this characterization.

Materials and methods

Both cell lines were derived from MPNSTs in a patient with NF1. A 30-year-old man, who had been diagnosed as having NF1 on the basis of clinical findings, was admitted to Niigata University Hospital because of a soft tissue tumour in the right thigh. Open biopsy revealed MPNST. Although the patient was treated with preoperative chemotherapy followed by radical surgery and postoperative chemotherapy, he died of multiple lung metastases 13 months after the first surgery. Anticancer agents used for this patient were Adriamycin, etoposide and ifosfamide, which were clinically effective in bringing about shrinkage of the tumour mass preoperatively.

The specimen for cultivation of NMS-2 cells was obtained from the tumour resected at the first surgical intervention. The specimen for NMS-2PC cells was obtained from further resection 9 months after the first. Each specimen was minced aseptically with ophthalmological scissors and was taken in small pieces by means of a pipette. Small fragments were seeded in a plastic culture dish (Corning, New York, N.Y.) and were fed with RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum (M.A. Bioproducts, Walkersville, Md.) and 200 μg/ml of kanamycin sulfate (Meiji Seika, Tokyo, Japan). All

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cultures were maintained at 37° C in a humidified incubator with a constant flow of 5% CO_2 in air. The medium was renewed every 2 days, and the cultured cells were passaged once a week by trypsinization. Mycoplasma contamination was checked by MycoTect kit (Gibco, Grand Island, N.Y.), and only mycoplasma-free cultures were used for experiments.

Cultured cells were observed with an inverted phase-contrast microscope. Pieces of both parental tumours and those transplanted into nude mice were fixed in 10% formaldehyde and embedded in paraffin. The cut sections were stained with haematoxylin and eosin. Formalin-fixed, paraffin-embedded sections of the parental and tansplanted tumours and ethanol-fixed cultured cells on Lab-Tek chamber slides (Nunc, Naperville, Ill.) were stained by an immunoperoxidase method using a biotin–streptavidin–peroxidase (BSA) kit (Nichirei, Tokyo, Japan). The primary antibodies used in this study were as follows: mouse monoclonal antibodies to human vimentin (V9; Dako, Copenhagen, Denmark), neuron-specific enolase (BBS/NC/VI-H14; Dako), CD57 (Leu 7; Becton-Dickinson, Mountain View, Calif.), α-smooth muscle actin (1A4; Dako), desmin (D33; Dako), cytokeratin (KL1; Immunotech, Marseille, France), epithelial membrane antigen (E29; Dako), muscle-specific actin (HHF35; Enzo, New York, N.Y.) and rabbit polyclonal anti-human S-100 protein (Dako).

For scanning and transmission electron microscopic examination, cells were prepared in a standard manner reported previously [15, 17].

To determine the growth rate, 1×10^5 viable cells were seeded in 35-mm culture dishes and harvested every 2 days.

For heterotransplantation, approximately 1×10^7 cultured cells in 0.2 ml of fresh culture medium were injected s.c. into athymic female nude mice (BALB-/C-nu/nu, Clea, Tokyo, Japan). The animals were sacrificed when the tumour became 2 cm or more in the greater dimension.

For chromosomal analysis, the cultured cells were treated with colchicine (0.2 μg/ml). Chromosome numbers were counted on 30 metaphases, and for karyotyping a trypsin-Giemsa banding method was used.

Chemosensitivity tests were carried out using a colorimetric MTT assay described by Mosmann [20]. Anticancer agents used in the present study were Adriamycin (Wako, Tokyo, Japan), etoposide (Wako) and ifosfamide (Shionogi, Osaka, Japan). Cells were seeded in 96-well microplates (Corning) at an initial density of 5,000 cells per well in 200 μl medium without anticancer reagents. After incubation for 48 h, anticancer agents were added at various concentrations. Cells were treated with anticancer agents for 2 h or 48 h. Then cells treated with anticancer agents for 2 h were incubated for 46 h with agent-free medium. After 48 h of treatment with anticancer agents, the medium in each well was replaced by 110 μl fresh medium containing MTT (1 mg/ml; Takara Biochemicals, Kyoto, Japan) and was incubated for 4 h. Thereafter, 100 μl isopropanol/HCl solution was added to each well to dissolve any formazan crystals that had developed. Absorbance of MTT in each well was measured immediately at 560 nm wavelength using a Tirtertek automated microtilter plate reader. Each experiment was performed in triplicate at least three times. Statistical differences were determined by means of an unpaired Student's *t*-test, a *P*-value of less than 0.01 being considered significant.

The induction of morphological differentiation was attempted with several agents including all-*trans*-retinoic acid (ATRA), dibutyryl cyclic AMP, prostaglandin E_1 and nerve growth factor. All agents were obtained from Sigma (St. Louis, Mo.). ATRA were dissolved in 95% ethyl alcohol, and the absence of any effects of the final concentration of ethyl alcohol on cell growth or morphology had been confirmed in a preliminary study. The other agents were dissolved directly in the medium. The medium containing test agents was renewed every 2 days. In addition, the levels of expression of type I and type II NF1 GAP-related domain (NF1-GRD) transcripts were determined by RT-PCR analysis as described by Nishi et al. [22]. Total cellular RNA was extracted using a QuickPrep total RNA extraction kit (Pharmarcia, Uppsala, Sweden). Total RNA was reverse transcribed using the BRL preamplification system (Gibco, Grand Island, N.Y.). The primers for PCR were designed as follows: NF1G.C.5′-CAGAATTCCCCCCTCAACTTCGAAGT-3′ and NF1G.D.5′-TGCGTGCTGCATCAAAGTTGCTTTTCAC-3′. PCR conditions were as follows: denaturation at 94° C for 4 min followed by 29 cycles of denaturation at

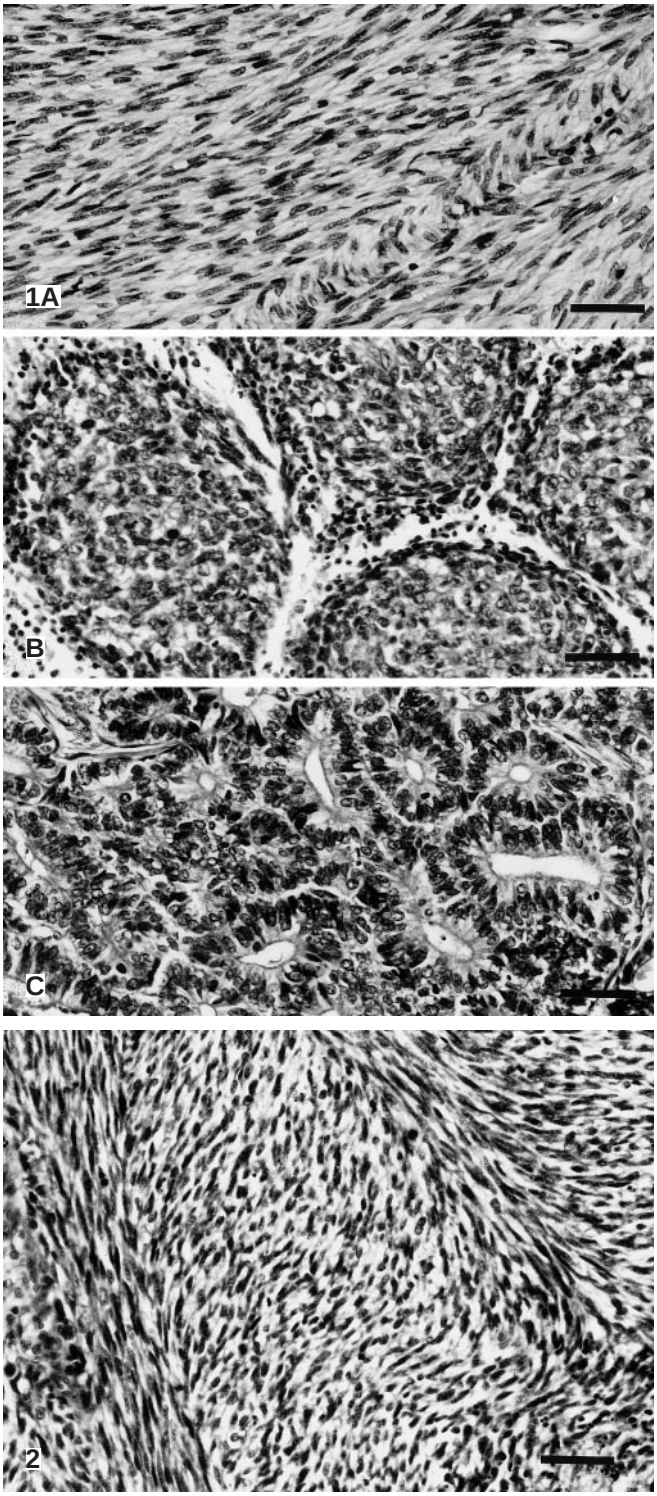

Fig. 1 The parent tumour of NMS-2 cells showed a histological combination of **A** fibrosarcomatous, **B** epithelioid and **C** glandular features. HE, ×185; *scale bars* 50 μm

Fig. 2 The parent tumour of NMS-2PC cells showed only typical feature of MPNST. HE, ×185; *scale bar* 50 μm

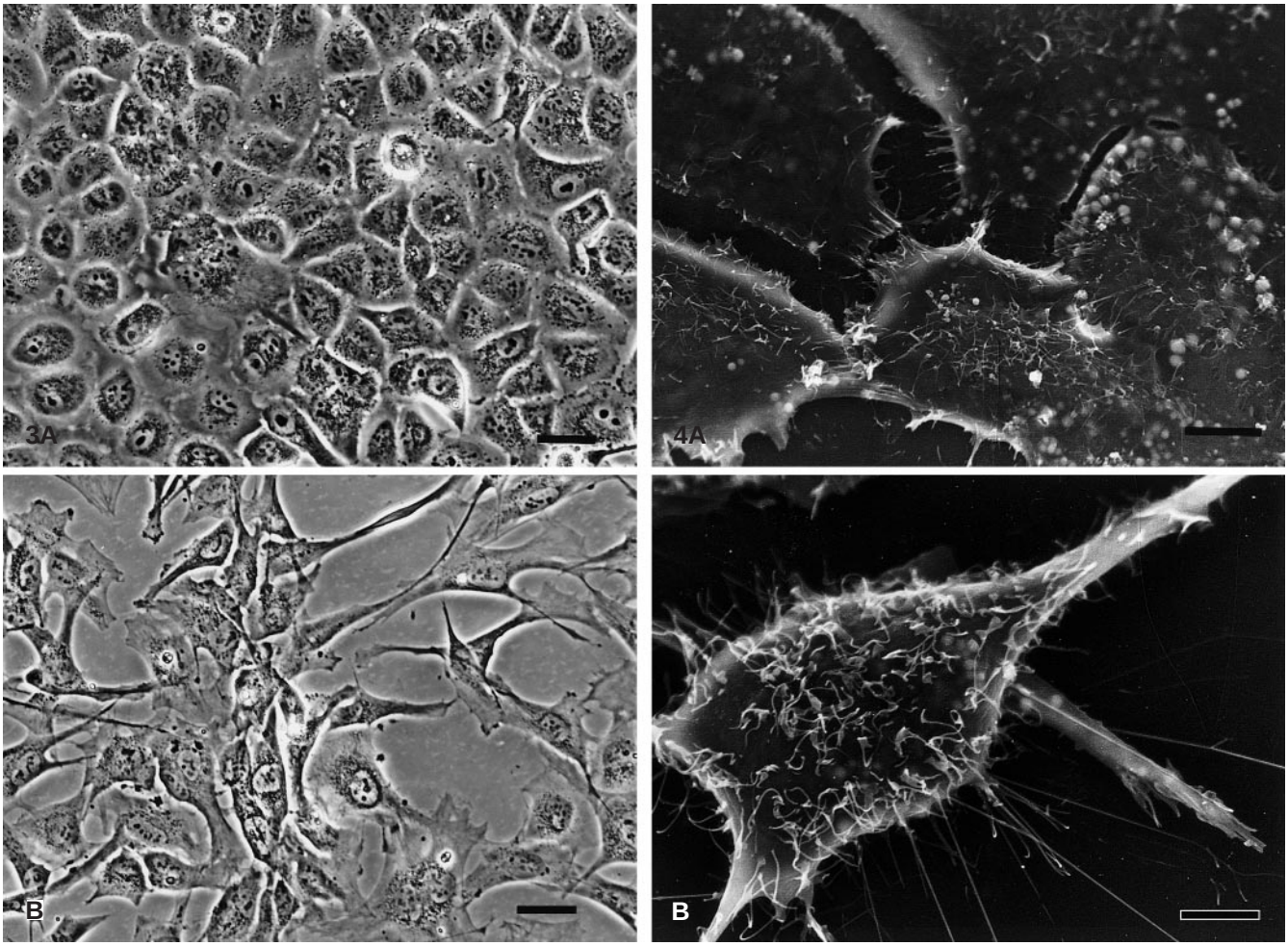

Fig. 3A Cultured NMS-2 cells grew in an epithelioid arrangement. **B** Cultured NMS-2PC cells exhibited fibroblast-like features. Phase contrast, ×740; *scale bars* 10 μm

Fig. 4 Scanning electron micrographs of **A** NMS-2 cells and **B** NMS-2PC cells. NMS-2 cells have short slender cytoplasmic processes. NMS-2PC cells have both long slender and thick cytoplasmic processes. ×1,980; *scale bars* 5 μm

93° C for 1 min, annealing at 60° C for 2 min, and extension at 72° C for 2 min, followed by a final extension at 72° C for 15 min. The PCR products were analysed by electrophoresis on 10% polyacrylamide gels and visualized by ethidium bromide staining. To quantify the relative levels of expression of type I (303-bp fragment) and type II (366-bp fragment) transcripts of NF1-GRD, the intensity of the ethidium bromide luminescence was measured with a CCD image sensor (Densitograph AE-6900-F; Atto, Tokyo, Japan). The results were determined as means±SE from three independent experiments. All testings were carried out between the 10th and 50th passages.

Results

The first tumour, which was the parent tumour from which NMS-2 cells were derived, showed a combination of three histological features; spindle, epithelioid and glandular MPNSTs (Fig. 1). The second tumour, from which NMS-2PC cells were derived, showed only typical features of MPNST similar to fibrosarcoma (Fig. 2). Cultured NMS-2 cells showed epithelioid features (Fig. 3A), while NMS-2PC cells were fibroblast-like (Fig. 3B). Scanning electron microscopic examination revealed that NMS-2 cells adhered directly or by relatively short slender cytoplasmic processes to one another (Fig. 4A), while NMS-2PC cells adhered to each other by long slender or thick cytoplasmic processes (Fig. 4B). Transmission electron microscopic examination revealed that the slender cytoplasmic processes contained microfilaments (Fig. 5A) and thick cytoplasmic processes mainly contained microtubules (Fig. 5B). The grafting success rates were 33.3%, with 3 or 9 mice taking NMS-2 cells, and 55.6%, with 5 of 9 mice taking NMS-2PC cells. All transplanted tumours showed the same histological features of typical MPNST and were composed of spindle-shaped cells. There were no significant histological differences between NMS-2 tumours and NMS-2PC tumours.

The population-doubling times of cell lines NMS-2 and NMS-2PC were 50.2 h and 44.7 h, respectively.

The results of immunocytochemical and immunohistochemical examinations are summarized in Table 1. Almost all NMS-2 and NMS-2PC cells were positive for S-10 protein. Vimentin was also detected in both cell lines.

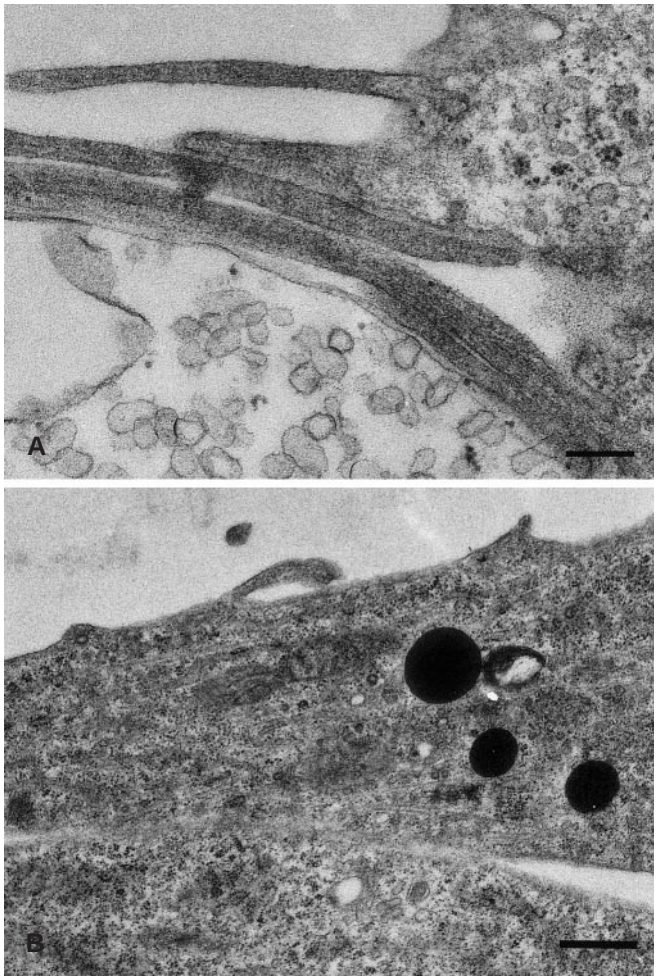

Fig. 5A, B Transmission electron micrographs of NMS-2PC cells. **A** Long slender cytoplasmic processes contain microfilaments; **B** thick cytoplasmic processes contain microtubules. **A** ×3,600; *scale bar* 2.5 μm **B** ×10,000; *scale bar* 1 μm

Chromosomal analysis of NMS-2 cells performed at the 15th passage revealed that the chromosome number ranged from 52 to 61, with a mode of 57. Cytogenetic analysis showed 57, X, –Y, +X, +add (X)(q13)×3, +add (1)(p11), add (1)(p11), add (1)(p11), add (2)(p25), der(2) t (1;2)(q12;q13), +3, add(4)(q11), add(5)(p11), add(5)(q11.2)×2, add(6)(q11), +add(7)(p22), +add (7)(p11.2), add (8)(p11.2), –9×2, add(10(q22), add(10)-(q11.2), add (11)(p15)×2, +add (15)(p11.2), –16×2, add (17)(p13q25), +add(17)(q25), add(17)(q11.2), add(17)-(q25), –19, –22, +3 mar. A chromosomal translocation, t (1;2), was detected. Chromosomal analysis of NMS-2PC cells performed at the 15th passage revealed that chromosome number ranged from 54 to 59, with a mode of 56. Cytogenetic analysis showed 56, X, –Y, +add (X)-(q13)×2, +add (1)(p11), add (1)(p11), add (2)(p25); +3, ins (5 ; ?)(q11.2 ; ?)×2, add (8)(p11.2)×2, –9×2, add (11)(p15)×2, +12, +add(13)(p11.2), +14, +add (15)-(11.2), +add (16)(p11.2), add (16)(q12.1)×2, add (17)-(q25)×2, add (18)(q21), –22×2, +4 mar. No chromosomal translocations were detected in the NMS-2PC cell line (Fig. 6).

Table 2 Anticancer agent sensitivities of NMS-2 and NMS-2PC cells

Anticancer agent	IC_{50}[a] for 2 h exposure		IC_{50} for 48 h exposure	
	NMS-2	NMS-2PC	NMS-2	NMS-2PC
Adriamycin	4.20±1.97	0.09±0.02*	0.88±0.07	0.07±0.01*
Etoposide	25.74±0.25	0.78±0.15*	6.70±0.44	0.31±0.11*
Ifosfamide	28.78±9.21	23.65±8.81	21.42±7.15	13.25±3.11

* $P<0.05$ compared with value for NMS-2 cells
[a] IC_{50} is the concentration (μg/ml) of that inhibits 50% of cell growth. Each value is the mean±standard error in IC_{50} found in three independent experiments

The results of chemosensitivity tests are shown in Fig. 7. The concentrations of anticancer agents at which 50% growth inhibition occurred (IC_{50}) are summarized in Table 2. NMS-2PC cells were significantly more sensitive to Adriamycin and etoposide than NMS-2 cells, both with 2 h and with 48 h exposure.

ATRA promoted morphological differentiation only in the NMS-2PC cell line. After 7 days of treatment with 1×10^{-6} M ATRA, NMS-2PC cells became flat and polygonal in shape, similarly to NMS-2 cells. However, no other agents had any effects on the morphological differentiation of either NMS-2 or NMS-2PC cells.

Table 1 Immunohistochemical properties of parent tumours, cultured cells and xenografted tumours (*NSE* neuron-specific enolase, *α-SMA* α-smooth muscle actin, *MSA* muscle-specific actin, *EMA* epithelial membrane antigen, *PoAb* polyclonal antibody, *MoAb* monoclonal antibody, – negative, + a few positive, ++ occasionally positive, ++ frequently positive)

Marker	Antibody type	Primary tumour		Second tumour	Cultured cell		Xenografted tumour	
		Spindle cell	Polygonal cell		NMS-2	NMS-2PC	NMS-2	NMS-2PC
S-100 protein	PoAb (rabbit)	++	+	+	+++	+++	+	+
NSE	BBS/NC/VI-H14, MoAb	–	–	–	–	–	–	–
CD57	Leu 7, MoAb	–	–	–	–	–	–	–
Vimentin	V9, MoAb	+++	+	+	+++	+++	+	+
Desmin	D33, MoAb	–	–	–	–	–	–	–
α-SMA	1A4, MoAb	–	–	–	–	–	–	–
MSA	HHF35, MoAb	–	–	–	–	–	–	–
EMA	E29, MoAb	–	–	–	–	–	–	–
Cytokeratin	KL1, MoAb	–	–	–	–	–	–	–

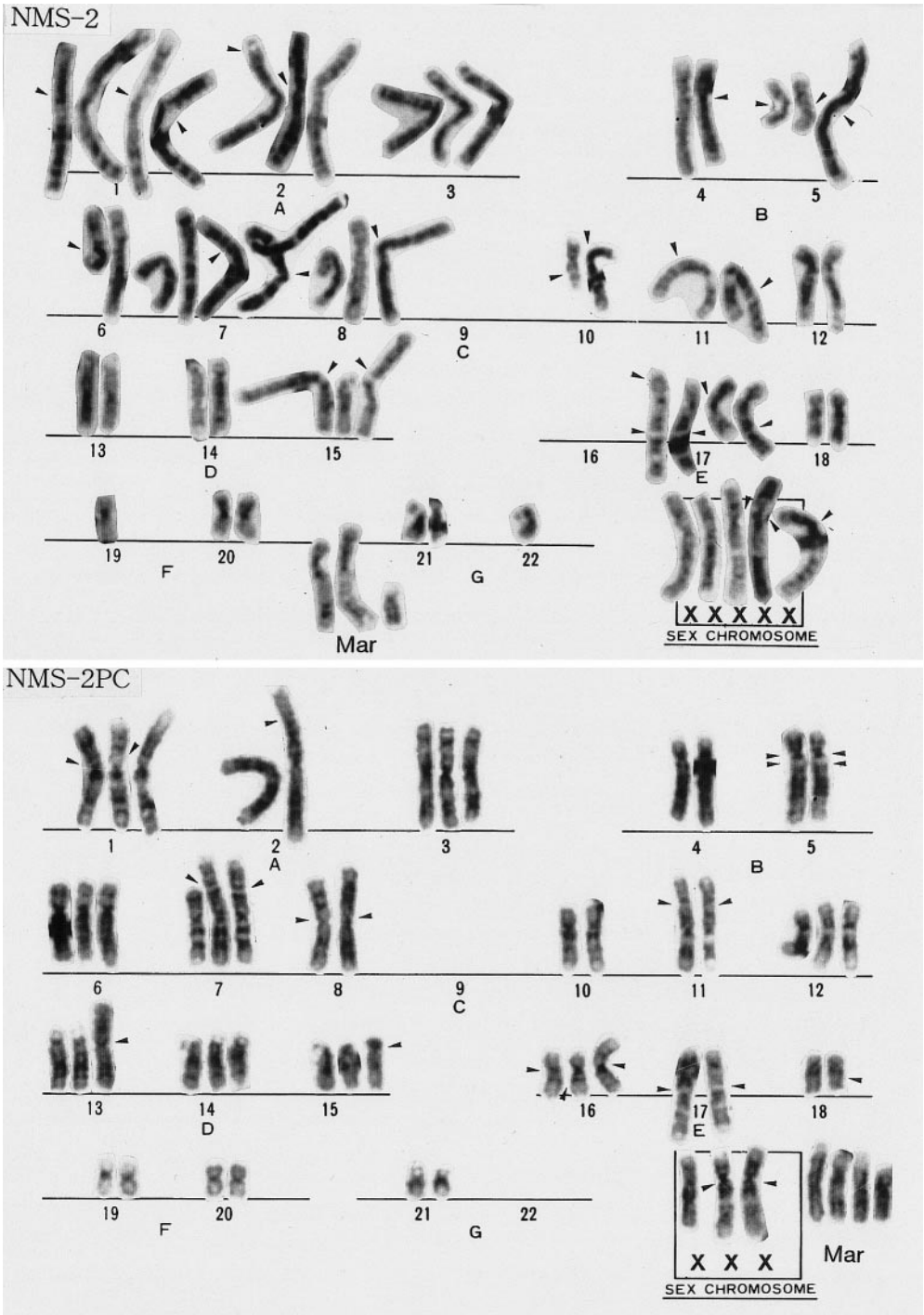

Fig. 6 The modal karyotypes of the NMS-2 and NMS-2PC cell lines. The NMS-2 cell line had a t (1; 2) chromosomal translocation

The ratios of type I/type II transcripts of NF1-GRD were calculated and are summarized in Fig. 8. The ratios of type I/type II were 0.745±0.106 and 0.395±0.042 in NMS-2 and NMS-2PC cell lines, respectively. The ratio in NMS-2PC cells treated with ATRA increased a little during the course of the morphological differentiation.

Discussion

MPNSTs originate from the neural crest and are multipotential. Histological variations in cell differentiation have been reported with the Triton tumour, which shows rhabdomyoblastic differentiation [2, 3, 28], epithelioid differentiation [1, 6, 18] and glandular differentiation [27]. These variations are rare, especially glandular differentiation. Minor variations include bone or cartilage elements, squamous differentiation, cords or nests of

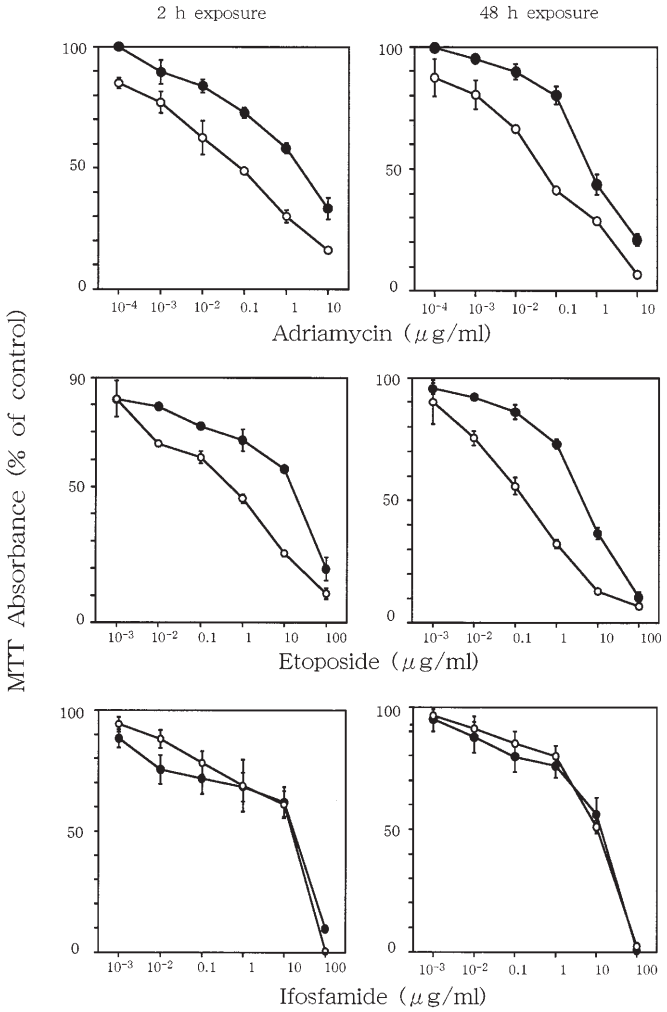

Fig. 7 Effects of exposure to three different anticancer agents for 2 h and 48 h (–●– NMS-2, –○– NMS-2PC)

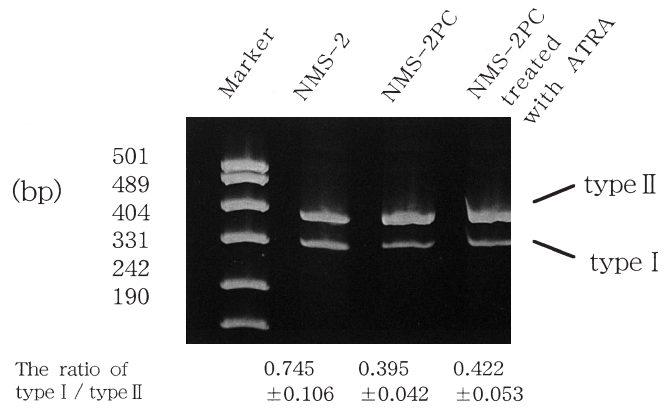

Fig. 8 Type I (303-bp) and type II (366-bp) transcripts of NF1-GRD detected by RT-PCR

small round cells and rosettes [9]. These unusual MPNSTs seem to occur more often in NF1 patients [12, 13]. The parent tumour from which NMS-2 cells were derived contained considerable numbers of epithelioid cells in addition to the typical MPNST pattern. We consider that the NMS-2 cells are derived from the epithelioid tumour cells of the parent tumour because of their appearance. MPNST cell lines reported previously have shown spindle cell features reminiscent of NMS-2PC cells in vitro [5, 21, 23]. NMS-2 seems to be a rare and unique MPNST cell line, which has an epithelioid appearance in vitro. NMS-2 and NMS-2PC cells had cytoplasmic processes and were positive for S-100. The cytoplasmic processes contained microtubules and microfilaments and extended from cell to cell. No special junctional structures or basal lamina were developed. In addition, transplanted tumours derived from NMS-2 cells in nude mice showed typical MPNST features. ATRA induced NMS-2PC cells to undergo epithelioid differentiation making the similar to the NMS-2 cells, suggesting that the two cell lines were derived from the same kind of cells, probably Schwann cells. Although the parent tumour of NMS-2PC developed after intensive chemotherapy with Adriamycin, etoposide and ifosfamide, chemosensitivity tests indicated, surprisingly, that NMS-2PC cells were far more sensitive to Adriamycin and etoposide than NMS-2 cells.

In some neuroblastoma cells, the ratio of type I/type II transcripts of NF1-GRD was changed when neuronal differentiation progammes were induced by ATRA [22]. However, the ratio changed little in NMS-2PC cells treated with ATRA. This functional differentiation may be delayed relative to morphological differentiation in Schwann cells. S-100 protein was not frequently detected in the parent or xenografted tumours, as shown in Table 1. In the MPNST, functional expression also varied between cells.

Recent cytogenetic analyses of MPNSTs reveals complex karyotypic abnormalities of several chromosomes, such as chromosomes 1, 11, 12, 14, 17 and 22 [10, 11, 16, 19], but specific translocations are not reported. Jhanwar et al. [16] indicated that an abnormality of chromosome 17 was seen in all their ten MPNST cases, and the loss of chromosome had 17q11.2, to which the NF1 gene has been localized, in five MPNSTs. In this study NMS-2 and NMS-2PC both had complex karyotypic abnormalities, including some of chromosome 17.

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