Virchows Archiv A Pathol Anat (1991) 418:333–338

Virchows Archiv A
Pathological Anatomy and Histopathology

N-*myc* gene amplification and neuron specific enolase production in immature teratomas

Isamu Ishiwata[1], Chieko Ishiwata[1], Masayuki Soma[1], Isao Ono[2], Takenori Nakaguchi[2], Kensuke Joh[3], Masakuni Furusato[3], and Hiroshi Ishikawa[4]

[1] Ishiwata Obstetric and Gynecology Hospital, 1-4-21, Kami-Mito, Mitoshi, Ibaraki-ken 310, Japan
[2] Mutual Aid Association, Suifu Hospital, 2-1-40, Omachi, Mitoshi, Ibaraki-ken 310, Japan
[3] Department of Pathology, [4] Department of Anatomy, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105, Japan

Received August 25, 1990 / Accepted November 16, 1990

Summary. The primary tumour tissues from 13 teratomas were investigated, 3 cases of immature teratoma (1 of pure type and 2 of mixed type), 5 cases of dermoid cyst, and 5 cases of mature solid teratoma, with special reference to N-*myc* gene amplification, productivity of neuron specific enolase (NSE), and the presence of double minutes (DMs). The possible relationship between these variables and malignancy of the tumours was also examined. N-*myc* gene amplification and NSE production were recognized in the primary tumour tissues and the first and the third passage cultured cells of all of the 3 cases of immature teratoma containing immature neural tissues. In 2 cases DMs were recognized. In dermoid cysts and mature teratoma, neither N-*myc* gene amplification nor NSE production were recognized in either the primary tumour tissues or cultured cells. The chromosomes were normal. Malignancy of teratoma is generally decided by the clinical stage and histological grade, but a more securely based decision is necessary where an immature teratoma contains immature neural tissues. The presence of N-*myc* gene amplification, NSE productivity and the presence of DMs may be valuable.

Key words: Teratoma – N-*myc* gene amplification – Neuron specific enolase

Introduction

Amplification of oncogenes takes place in some tumours and a relationship between gene amplification and tumour malignancy has been suggested. Amplification of the N-*myc* gene is found in certain types of tumour of neural and neural crest origin, and many detailed reports are available, especially on the effect of malignancy of neuroblastoma produced by gene amplification (Brodeur et al. 1984). A poorer prognosis is expected for neuroblastomas with gene amplification than those without. It has also been shown that N-*myc* gene amplification takes place on particular chromosomes, such as double minutes (DMs) and homogeneously staining regions (HSR) (Kohl et al. 1983).

Immature teratoma often contains immature neural elements and the prognosis is closely related to the histological grade (Nogales et al. 1976), but a more comprehensive prognostic index is necessary similar to N-*myc* gene amplification of neuroblastoma. We therefore examined, in teratoma containing neural components, the occurrence of N-*myc* gene amplification, the tissue component where amplification occurs, the presence of DMs and HSR, and the malignancy of the tumours.

Offprint requests to: H. Ishikawa

Materials and methods

The primary tumour tissues and the cultured cells used were derived from 3 cases of immature teratoma, 5 cases of dermoid cyst and 5 cases of mature solid teratoma, as shown in Table 1. The tumours were graded from 1 to 3 according to the criteria of Thurlbeck and Scully (1960). Benign solid or cystic teratomas were graded 0. The tissues were rinsed twice with culture medium (Ham's F-12 containing 15% fetal calf serum; Gibco, Gland Island, N.Y.), minced with a sharp pair of scissors, digested with 600 pronase units (PU) Dispase/ml (Gohdo Shusei, Tokyo) for 15 min at 37° C, and centrifuged at 300 *g* for 10 min. The sediments were resuspended in growth medium, placed in 6-cm plastic dishes (Terumo, Tokyo), and cultured in a 5% CO_2 incubator at 37° C. The cells were dispersed using 600 PU Dispase/ml and cultures were made at a 1:2 dilution. The primary tumour tissues (culture materials) were fixed in 10% buffered formalin, embedded in paraffin, cut at 4 μm, and finally stained with haematoxylin and eosin. These tissues were also immunostained with anti-neuron-specific enolase (NSE), anti-S-100 protein (P), anti-alpha-fetoprotein (AFP), anti-carcinoembryonic antigen (CEA) and anti-human chorionic gonadotropin (hCG) (all antibodies from Dako Immunoglobulins, Copenhagen, Denmark) by the method of Sternberger et al. (1970) using peroxidase anti-peroxidase (PAP) complex. The cultured cells were observed under a phase contrast microscope, fixed with 95% ethanol, and stained with Papanicolaou solution. Some cultured cells were also fixed with 10% buffered formalin and immunostained with the above-mentioned antibodies. Also, 5×10^5 cells were cultured in a serum-free Ham's F-12 and the concentration of such tumour markers as NSE, AFP, CEA, hCG, carbohydrate

Table 1. Biological properties of teratomas (pure and mixed type)

Case	Age	Sex	Organ	Histology	N-*myc* amplification		Chromosome
					PT	Cul C	
1	28 years	F	Ovary	IMT+AC	×50	×50	Diploid+M
2	22 years	F	Ovary	IMT	×50	×30	Diploid+M+DMs
3	21 years	M	Testis	IMT+SEM+YST+CC	×30	× 5	Tetraploid+M+DMs+HRS
4	23 years	F	Ovary	DC	–	–	Diploid
5	35 years	F	Ovary	DC	–	–	Diploid
6	36 years	F	Ovary	DC	–	–	Diploid
7	37 years	F	Ovary	DC	–	–	Diploid
8	48 years	F	Ovary	DC	–	–	Diploid
9	5 months	M	Stomach	MT	–	–	Diploid
10	4 months	F	Retroperitoneum	MT	–	–	Diploid (normal)
11	2 months	F	Sacrococcygeum	MT	–	–	Diploid (normal)
12	1 year	M	Retroperitoneum	MT	–	–	Diploid (normal)
13	3 days	F	Sacrococcygeum	MT	–	–	Diploid (normal)

IMT, Immature teratoma; AC, anaplastic carcinoma; SEM, seminoma; YST, yolk sac tumour; CC, choriocarcinoma; DC, dermoid cyst; MT, mature teratoma; PT, primary tumour tissue; Cul C, cultured cells; chromosome M, marker chromosome; DMs, double minutes; HSR, homogeneously staining region; NSE, neuron specific enolase; AFP, alpha-feto-protein; CEA, carcino-embryonic antigen; hCG, human chorionic gonadotropin; CA125, carbohydrate antigen 125; SCC, squamous cell carcinoma antigen; TPA, tissue polypeptide antigen; ND, not detected; NT, not tested

antigen 125 (CA125), squamous cell carcinoma antigen, and tissue polypeptide antigen was measured by radio-immunoassay 2 days later.

For chromosome analysis the cultured cells were treated with 1×10^{-7} M Colcemid for 4 h and treated with 0.1% trypsin solution for 15 s at room temperature, stained with 3% Giemsa solution (pH 6.8), and analyzed for G-band karyotyping (especially, HSR and DMs).

To evaluate N-*myc* gene amplification samples (4 μg) of DNA from each culture material (primary tumour tissue) and the cell line were digested to completion with *Eco*RI, subjected to electrophoresis on a 0.8% agarose gel, and transferred to nitrocellulose filter (Southern et al. 1975). DNA from human placenta and neuroblastoma cell line (IMR32) (Kanda et al. 1987) were used as negative (single copy) and positive controls (50 fold amplification of N-*myc*) respectively. The presence or absence of N-*myc* gene amplification, which was represented by a 2-kb *Eco*RI fragment, was examined with the Southern blotting method using the isotopically labelled probe NB-19-21 (Kohl et al. 1984). N-*myc* copy number was determined by densitometric analysis.

Results

The histological characteristics of each culture from the primary tumour tissue are shown in Table 1. The tissues of all the three immature teratomas were composed of organoids containing structures similar to immature alimentary canal, choroid flexus, neural canal, neural tissue, bone, cartilage, epidermis and hair follicles. Immature nervous tissues were contained in all the three immature teratomas (cases 1, 2 and 3; grade 3; (Fig. 1)). NSE and S-100P were detected immunohistochemically (especially in the immature neural tissue). AFP, CEA and hCG were also detected in yolk sac tumour, immature alimentary canal and choriocarcinoma, respectively.

Initially in the primary culture of all immature teratomas, squamous epithelial cells, columnar epithelial cells, fibroblasts and nerve cells were detected in the phase contrast microscope. The epithelioid cell line (HUOT) (Fig. 2A) was established from case 1 and it was found to produce AFP (Ishiwata et al. 1985b). The immature neurogenic cell line (HTOMT) (Fig. 2B) producing NSE, and the choriocarcinoma cell line (HKRT-II) (Fig. 2C) producing hCG were also established from case 2 and case 3 respectively. Fibroblasts were found to grow only in the 5 dermoid cysts, though the cell line was not established. However, the totipotential stem-cell clonal strain (HOGT) was established from case 9 (Ishiwata et al. 1985a) and the fibroblast strains were established from cases 10–13 (mature solid teratoma). Nerve cells with cytoplasmic processes and containing NSE and S-100P were detected in the cultures (within three passages) of immature teratomas, but they had disappeared in the five passages of HUOT and HKRT-II lines. NSE was detected in the conditioned media of all three immature teratomas, but it decreased with subculturing and was not detected after 5 passages (Table 2). The tumour markers in the conditioned media of these cultures are shown in Table 1.

The results of G-band karyotyping of cultures are shown in Table 1. The cells did not show a karyotypic abnormality upon G-band karyotyping except for immature teratomas. The chromosome number of immature teratomas varied over a wide range and a majority of the cells showed karyotypic abnormality. The DMs were detected in 20% of HTOMT cells and 10% of HKRT-II cells (Fig. 3), but not in HUOT cells. HSR was detected in the HKRT-II line.

The N-*myc* gene was amplified in the primary tumour tissues and the cultured cells (within three passages) of all three immature teratomas. It was amplified around 50-fold (HUOT line), 30-fold (HTOMT), 5-fold (HKRT-II) in the three passaged cells and about 50-fold (IMR32; positive control) (Fig. 2). The N-*myc* amplifi-

Tumour marker in primary culture							Cell line	Cell shape
NSE (ng/ml)	AFP (ng/ml)	CEA (μg/ml)	hCG (min/ml)	CA125 (U/ml)	SCC (ng/ml)	TPA (U/ml)		
45	210	ND	5	ND	ND	NT	HUOT	Epithelioid
880	29	28	5	1400	93	6700	HTOMT	Neuroblast
24	42	32	860	42	NT	NT	HKRT-II	Epithelioid
ND	ND	ND	ND	ND	ND	ND	Not established	
ND	ND	ND	ND	ND	ND	ND	Not established	
ND	ND	ND	ND	ND	ND	ND	Not established	
ND	ND	ND	ND	ND	ND	ND	Not established	
ND	ND	ND	ND	ND	ND	ND	Not established	
ND	ND	ND	ND	ND	ND	ND	HOGT	Fibroblast-like
ND	ND	ND	ND	ND	ND	ND	HGRT	Fibroblast
ND	ND	ND	ND	ND	ND	ND	HTST	Fibroblast
ND	ND	ND	ND	ND	ND	ND	HKRT-1	Fibroblast
ND	ND	ND	ND	ND	ND	ND	HSST	Fibroblast

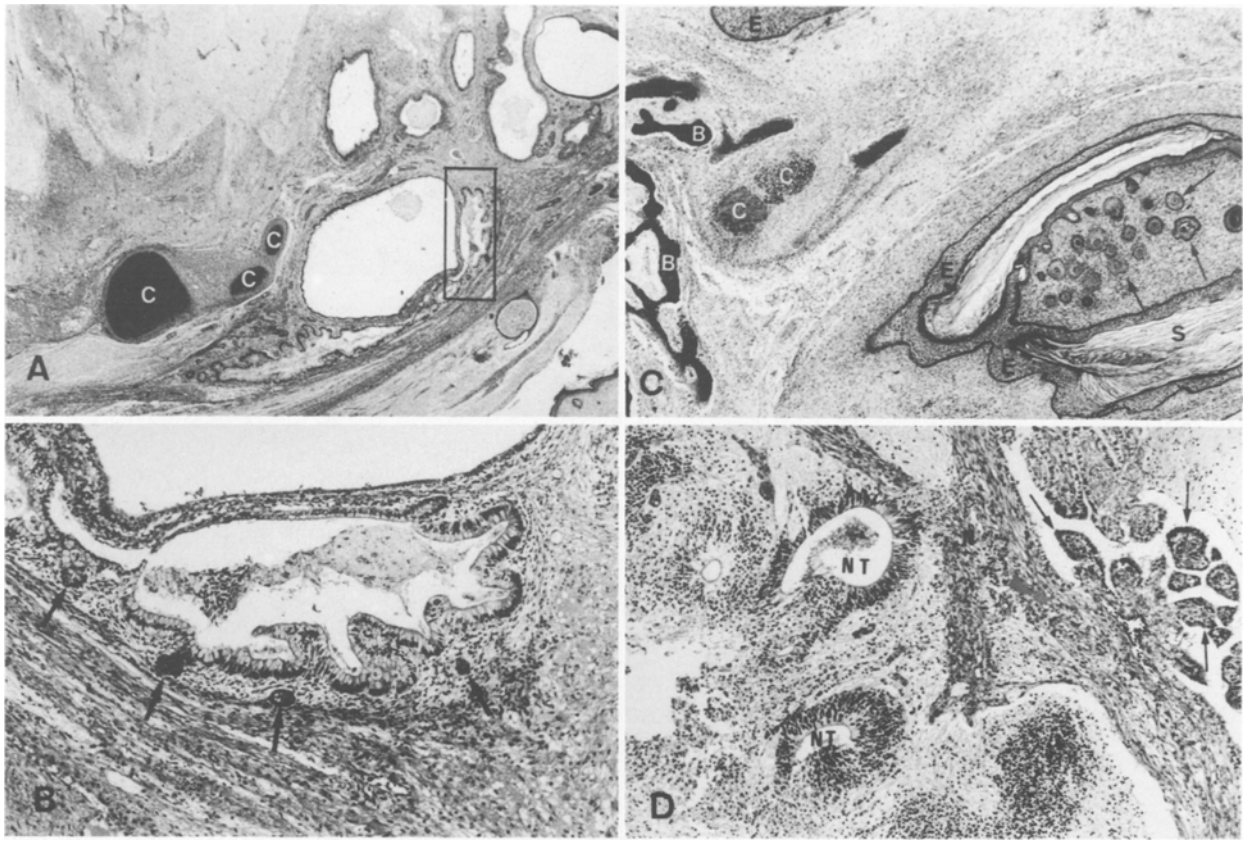

Fig. 1A–D. Ovarian teratoma (case 2). **A** Tridermal immature nature. **B** Marked area in **A** under higher magnification showing alimentary canal-like structure with numerous goblet cells in the epithelium and several glands (*arrows*) in the sub-epithelial region; **C** Skin-like and cornified structures and hair follicles (*arrows*). **D** Neural tube-like structure with chorioideus-like tissue (*arrows*). *B*, Bone; *C*, cartilage; *E*, epidermis; *NT*, neural tube-like tissue; *S*, stratum corneum. H&E, **A** ×10, **B, C, D** ×100

cation decreased with sub-culturing, and was not observed after five passages in the HUOT and HKRT-II lines, but it was observed to be stable in the HTOMT line. Amplification was not seen in any other teratoma in this study.

Discussion

Oncogenes are important in the control of proliferation and differentiation of organs in the fetus (embryogenesis) and generally show expression in the early stage of

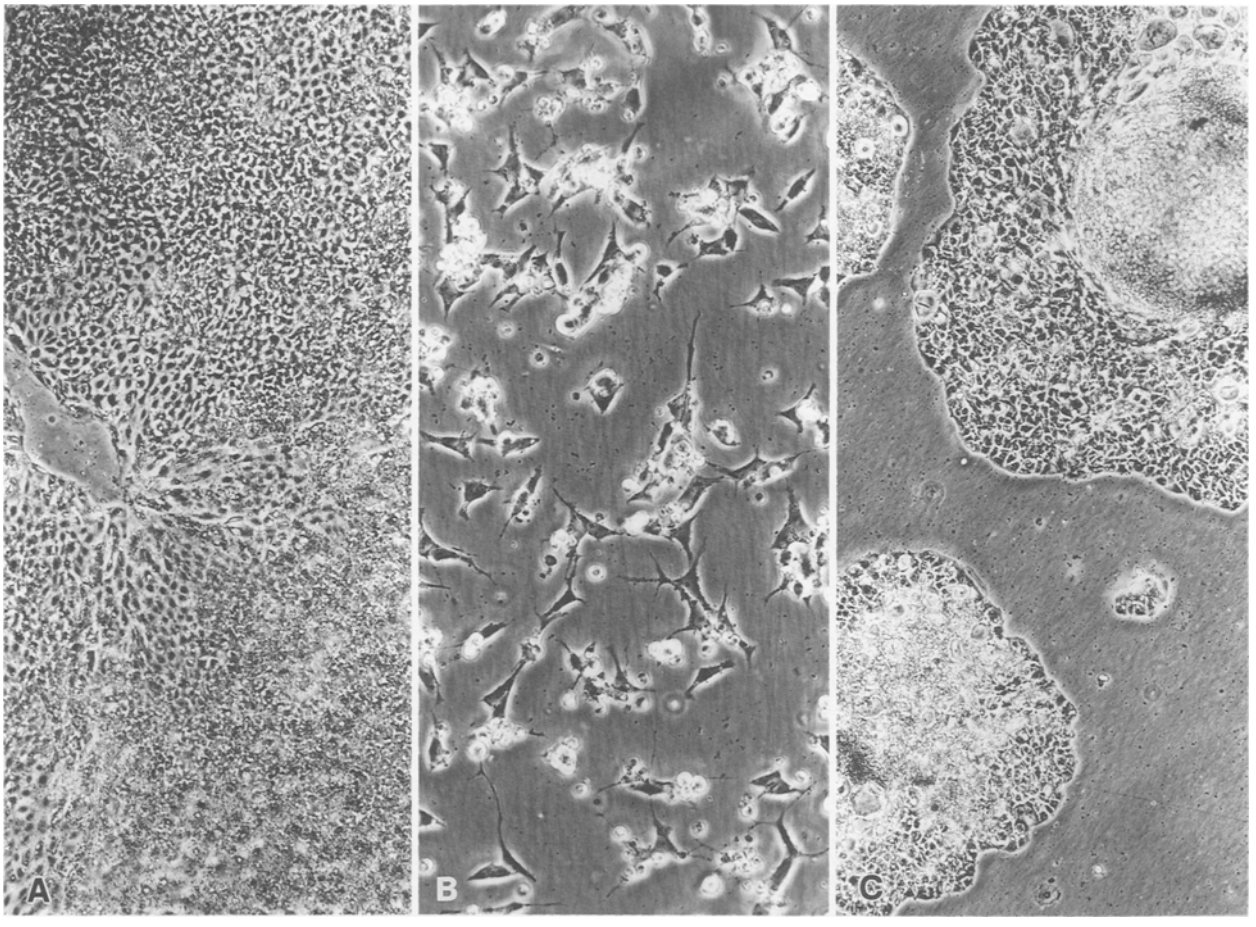

Fig. 2A–C. The cultured cells. **A** HUOT (case 1); **B** HTOMT (case 2); **C** HKRT-II (case 3). Phase contrast; **A**, ×40; **B**, ×100; **C**, ×40

Table 2. N-*myc* gen amplification of cultured cells and NSE concentrations in their conditioned media

Case	Cell line	Passage number	N-*myc* amplification	NSE (ng/ml)
1	HUOT	1	×60	62
		3	×50	45
		5	–	ND
		10	–	ND
		30	–	ND
2	HTOMT	1	×50	880
		3	×30	430
		5	×24	370
		10	×28	340
		30	×17	325
3	HKRT-II	1	× 5	24
		3	× 3	21
		5	–	ND
		10	–	ND
		30	–	ND

ND, Not detected

development. Gene amplification is observed in several kind of tumours including amplification of c-Ki-*ras* in lung, colon, gastric, and ovarian cancers; c-Ha-*ras* in malignant melanoma and bladder cancer, c-*myb* in acute myeloblastic leukaemia and colon cancer, c-*abl* in chronic myelocytic leukaemia, and c-*myc* in Burkit's lymphoma, the pro-myelocytic leukaemia cell line (HL-60), colon, lung, breast, and gastric cancers (Kanda 1987). N-*myc* is the 2.0 kb *Eco*RI fragment of genomic DNA, which has been separated from human neuroblastoma cell line and exhibits a 78% homology with exon 2 of the human c-*myc* gene (Schwab et al. 1983). The position of the N-*myc* gene in normal cells is chromosome 2*p*23-24, but N-*myc* gene amplification takes place on DMs and HSR as revealed by in situ hybridization (Kohl et al. 1983). N-*myc* gene-amplification is reported in neuroblastoma, retinoblastoma and small cell carcinoma of lung. Amplification of N-*myc* in neuroblastoma is peculiar; and its frequency is reported to be higher with advancing stage of the tumour. N-*myc* gene amplification is found in 50% of cases in stage III to IV, but rarely in those in stage I or II. Even if the tumours are in the same stage, a poorer prognosis is expected for those with gene amplification than those without. These facts make us believe that neuroblastoma acquires a strong proliferating and metastasizing capacity by N-*myc* gene amplification. In fact, it is easy to establish a cell line of neuroblastoma with N-*myc* amplification; and it is also easy to transplant these cells onto a nude mouse (Kanda et al. 1987).

NSE exists in neural cells and neuroendocrine cells, but is present at a high level in the blood of patients

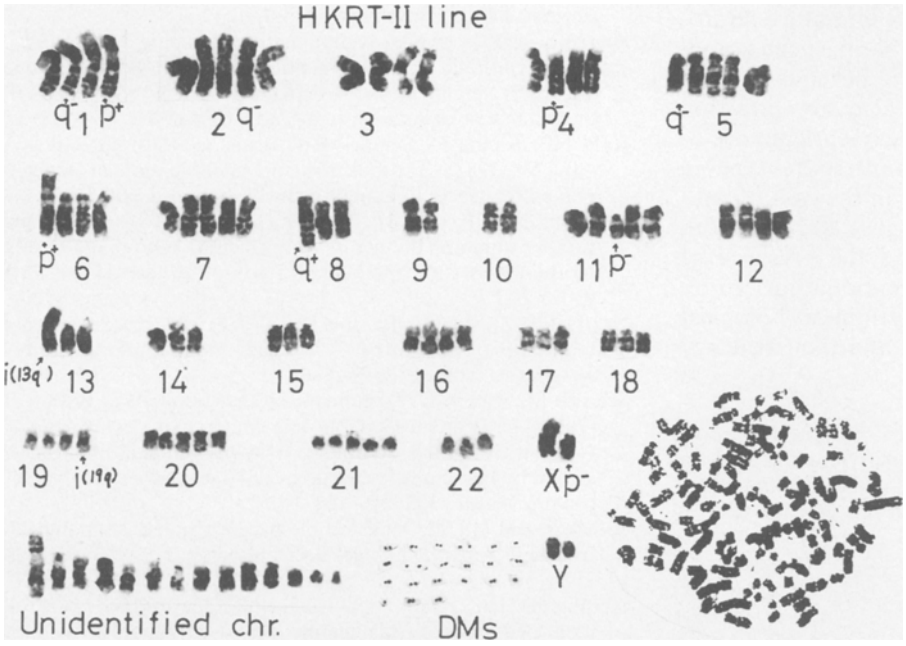

Fig. 3. Metaphase plate and G-band karyotype of HKRT-II cell (passage 10) showing a large number of double minute chromosomes

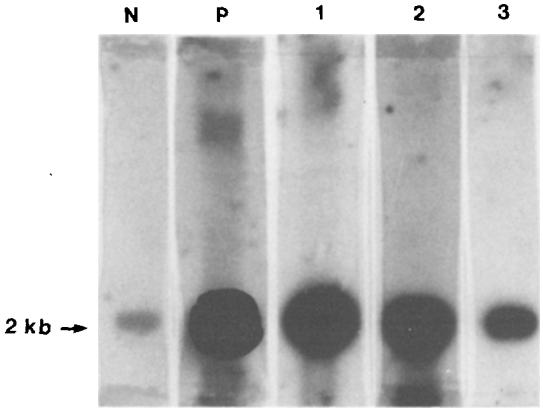

Fig. 4. Autoradiograph of DNA from three cases of immature teratomas. Samples of human placental DNA were used as a measure of single-copy intensity (*N*), and DNA from IMR32 cells (*P*), which has around 50 fold amplification of N-*myc*, was used as a positive control. The N-*myc* amplified approximately 50-fold in HUOT (*1*), 30-fold in HTOMT (*2*), and 5-fold in HKRT-II (*3*)

with neuroblastomas, where it is used as a tumour marker (Zeletzer et al. 1983).

Immature neural components are often included in immature teratoma, and the value of a histological grading based on the mitotic activity and maturation of the tissue as a prognostic index has been supported by many investigators (Nogales et al. 1976; Norris et al. 1976). These facts led us to examine (1) N-*myc* amplification, (2) production of NSE, a tumour marker of neural tissue, (3) presence of DMs, etc. in immature teratoma, dermoid cyst, and mature teratoma containing neural component. We also attempted to culture their primary tumour tissues and to establish cell lines derived from the neural elements. Only proliferation of fibroblasts was observed in tissue culture of dermoid cyst. N-*myc* gene amplification and NSE production was observed neither in the cultured cell nor in the primary tumour tissue. In the tissue culture of solid mature teratoma, squamous epithelial cells, columnar epithelial cells, fibroblasts and nerve cells were found as a mixture, but no N-*myc* gene amplification was recognized in either the cultured cells or the primary tumour. In contrast, in tissue culture of immature teratoma (case 2; ovarian tumour composed of embryonal tissue), proliferation of fibroblasts, epithelial cells and neuroblasts was observed and N-*myc* gene amplification was recognized only in nerve cells at around 30-fold. Culture of nerve cells was successful with fast proliferation and long-term passage (30 times) to give HTOMT line. N-*myc* gene amplification and NSE production were observed with stability. DMs were recognized in the cells. Case 3 was a tumour of the testis with seminoma, yolk sac tumour, choriocarcinoma and immature teratoma present as a mixture; nerve cells and choriocarcinoma cells showed proliferation on culture; and amplification of N-*myc* and NSE production were observed in the nerve cells. Unfortunately the nerve cell system could not be established as a line. The choriocarcinoma line (HKRT-II) was, however, established and it produces hCG but not NSE in the in vitro system. HKRT-II cells were successfully transplanted on nude mouse to form choriocarcinoma (in preparation). In case 1 (ovarian tumour), an immature teratoma and an anaplastic epithelioid tumour (maybe atypical embryonal carcinoma) were present as a mixture, and N-*myc* gene amplification and NSE production were recognized up to the third cell passage, but they disappeared along with the cells of nervous origin. No DMs were recognized. A teratocarcinoma cell line producing AFP was established from the system, but differentiation into neural component and N-*myc* gene amplification were not re-

cognized. The results of our investigation show that N-*myc* gene amplification takes place in immature teratoma but not in mature teratoma, that N-*myc* gene amplification is recognized in the immature nervous component, and that cells showing N-*myc* gene amplification produce NSE and possess DMs. Consequently, in deciding malignancy of immature teratoma (pure type in case 2), it is necessary to consider, as in the case of neuroblastoma, the occurrence of N-*myc* gene amplification, the amount of NSE production, and the presence of DMs. This is in addition to the conventional procedure of considering the clinical stage, the pathomorphological immaturity of the neural component and the frequency of mitosis.

Acknowledgements. We thank Dr. D.R. Naik, Visiting Professor, Department of Anatomy, The Jikei University and Dr. Damon C. Herbert, Associate Professor, Department of Cellular and Structural Biology, The University of Texas, Health Science Center, for editing this manuscript.

References

Brodeur GM, Seeger R, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124

Ishiwata I, Ishiwata C, Mukai M, Ono I, Ohara S, Konoe K, Ishikawa H (1985a) Establishment and characterization of a new clonal strain derived from human benign gastric teratoma. Exp Pathol 27:143–151

Ishiwata I, Ishiwata C, Soma M, Tomita K, Nozawa S, Ishikawa H (1985b) Establishment and characterization of HUOT, a human ovarian malignant teratoma cell line producing alpha-fetoprotein. J Natl Cancer Inst 75:411–422

Kanda N (1987) Oncogene amplification. Oncologia 20:58–67

Kanda N, Tsuchida Y, Hata J, Kohl NE, Alt FW, Latt SA, Utakoji T (1987) Amplification of IMR-32 clones 8, G21 and N-*myc* in human neuroblastoma xenografts. Cancer Res 47:3291–3295

Kohl NE, Kanda N, Schreck RR, Bruns G, Latt SA, Gilbert F, Alt FW (1983) Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35:359–367

Nogales FFJR, Favara BE, Major EJ, Silverberg SG (1976) Immature teratoma of the ovary with a neural component ("solid" teratoma). A clinicopathologic study of 20 cases. Hum Pathol 7:625–642

Norris HJ, Zirkin HJ, Benson WL (1976) Immature (malignant) teratoma of the ovary. A clinical and pathologic study of 58 cases. Cancer 37:2359–2370

Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert E, Brodeur G, Goldstein M, Trent J (1983) Amplified DNA with limited homology to c-*myc* cellular oncogenes in shared by human neuroblastoma cell lines and a neuroblastoma tumors. Nature 305:245–248

Southern EM (1975) Detection of specific sequences among DNA fragment: separated by gel electrophoresis. J Med Biol 95:503–513

Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horse-radish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333

Thurlbeck WM, Scully RE (1960) Solid teretoma of the ovary. A clinicopathological analysis of 9 cases. Cancer: 804–811

Zeletzer PM, Marangos PJ, Parma AM, Sather H, Dalton A, Hammond D, Siegel SE, Seeger RL (1983) Raised neuron-specific enolase in serum of children with metastatic neuroblastoma. Lancet II:361–363