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Characteristics of rhabdomyosarcoma cell lines derived from uterine carcinosarcomas

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Abstract Rhabdomyosarcoma (RMS) is occasionally found in the female genital tract, and mostly appears as one of the heterologous mesenchymal components in uterine carcinosarcoma designated as malignant mixed müllerian tumour (MMMT). We examined the biological properties of a pure rhabdomyosarcoma (RMS) cell line designated FU-MMT-3, which was newly established from a surgical specimen taken from a patient with uterine MMMT. We also evaluated c-*myc* and MYCN gene amplification in three RMS cell lines (including FU-MMT-3) derived from three MMMTs by Southern blot analysis. FU-MMT-3 cells were propagated continuously for 57 serial passages over a 2-year period in vitro. FU-MMT-3 was able to produce tumours demonstrating pure RMS in athymic nude mice. Cytogenetically, FU-MMT-3 showed a triploidy pattern, with complex karyotypic abnormalities including trisomy of chromosome 8. All three RMS cell lines, including FU-MMT-3, showed amplification of the c-*myc* gene (approximately fourfold to eightfold), while no cell lines demonstrated MYCN gene amplification. FU-MMT-3 is considered to provide a useful system for the study of the biological behaviour of RMS in MMMTs. Extra copies of chromosome 8 and c-*myc* gene amplification may be associated with the rhabdomyoblastic differentiation in MMMT.

Key words Malignant mixed müllerian tumour · Carcinosarcoma · Rhabdomyosarcoma · Tissue culture · Cytogenetics

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Introduction

Rhabdomyosarcoma (RMS), a malignant mesenchymal tumour, accounts for approximately 5% of solid tumours in childhood and 55% of soft tissue sarcomas in children and adolescents [37]. Interestingly, however, these tumours are also occasionally found in the female genital tract, mostly as one of the heterologous mesenchymal components of uterine carcinosarcoma, designated as malignant mixed müllerian tumour (MMMT) [1, 3, 6, 20, 25, 29, 33, 34, 38, 39, 41, 44]. Most of these tumours have a highly aggressive behaviour and a poor prognosis, and many cytogenetic and molecular biological analyses of RMS in soft tissue have been published [8–13]. There have only been three reports, including our own, on the RMS of MMMT [14, 15, 32].

In our previous study both MMMT cell lines with rhabdomyoblastic features had extra copies of chromosome 8 [14]. We also demonstrated amplification of c-*myc* gene both in cultured MMMT cells and in the original tumour [15].

The objective of this study was to clarify the cytogenetic and molecular biological characteristics of RMS in MMMT by analysing a newly established RMS cell line, FU-MMT-3, which was derived from a patient with heterologous MMMT of the uterus. In addition, we also evaluated the genetic characteristics common to our three RMS cell lines and we discuss the relationship between rhabdomyoblastic differentiation in MMMT and c-*myc* gene amplification or extra copies of chromosome 8.

Case report

The patient was a 62-year-old postmenopausal Japanese woman diagnosed as having uterine sarcoma by an endometrial biopsy. She underwent total hysterectomy, bilateral salpingo-oophorectomy, and omentectomy for the uterine malignancy in February of 1990. She had received neither chemotherapy nor radiotherapy before the surgical treatments. The surgical material from the primary tumour

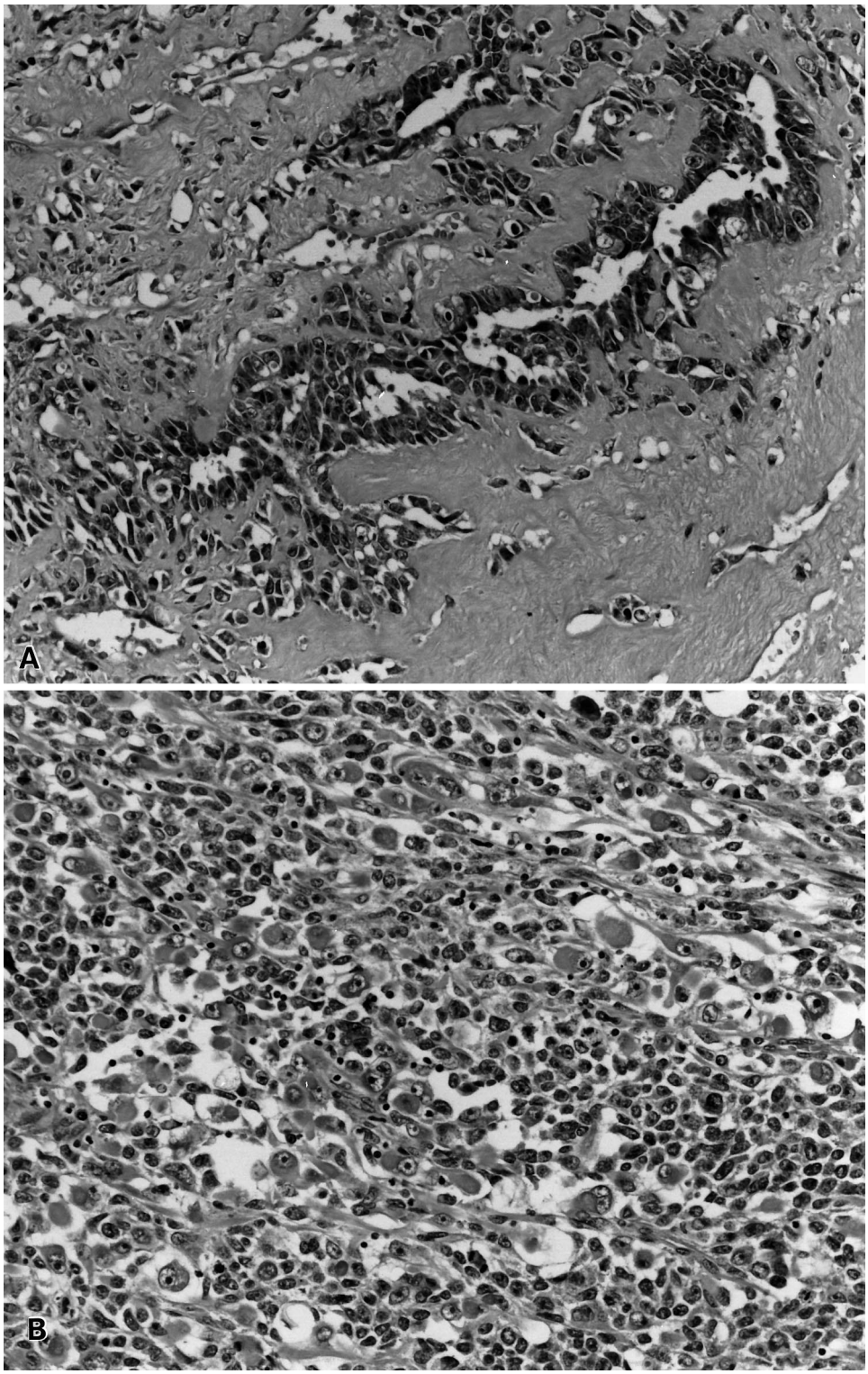

Fig. 1A, B Histology of the original tumour of FU-MMT-3. The tumour consists of a mixture of adenocarcinoma (**A**) and rhabdomyosarcoma (**B**). (**A**) Carcinomatous element shows malignant glandular structures (H & E, ×190). (**B**) Sarcomatous element demonstrates rhabdomyoblastic differentiation (H & E, ×190)

showed a mixture of endometrial adenocarcinoma and RMS, but no other heterologous elements were recognized. The tumour was composed chiefly (over 95% of the neoplasm) of RMS (Fig. 1A, B). Metastatic lesions in the abdominal cavity were also removed at surgery and consisted of RMS alone. Although she underwent eight courses of postoperative systemic multidrug chemotherapy (cisplatin-based), the patient died after a local recurrence and distant metastases developed 32 months after surgery.

Materials and methods

Immediately after the hysterectomy, tissue fragments obtained from primary tumours were mechanically dissociated and then digested with collagenage type II solution (200 IU/ml; Worthington Biomedical Co., N.J.) in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (Sigma, St Louis, Mo.). To obtain a single-cell suspension, the digestion was continued for

several hours in a humidified incubator at 37°C with 5% carbon dioxide and 95% air. The samples were centrifuged at 1,000 r.p.m. for 5 min, then were washed and resuspended in 20 ml of DMEM/F-12 after removing the supernatant.

The cells were planted in glass flasks (TD40, Ikemoto Rika, Tokyo, Japan) containing 10 ml of DMEM/F-12 supplemented with 10% fetal calf serum (FCS; Gibco) and kanamycin (100 μg/ml). The cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide in air. The medium was replaced twice a week. Confluent cell layers were obtained within 2 months of the primary cultures, and they were subcultured every 1 or 2 weeks at 1:2 or 1:4 dilution by using 0.1% trypsin and 0.02% EDTA solution. The main types of mycoplasmas were checked by a Myco Tect Kit (Gibco) and were all negative in each line.

The growth curve of the cultured cells at passage 5 was estimated by seeding 1×10^4 cells in each of five culture dishes (30-mm; Falcon 3001, Becton Dickinson, Oxnard, Calif.) containing 2 ml of DMEM/F-12 with 10% FCS. The cells in the culture dishes were harvested by using trypsin-EDTA and then resuspended in DMEM/F-12. The number of viable cells was counted by dye exclusion (0.1% trypan blue in PBS) every 24 h for 4 days.

The cells grown in culture flasks were observed by phase-contrast microscopy. For routine light microscopy, the cells cultured in chamber slides (Lab-Tek, Miles Laboratories, Naperville, Ill.) were fixed in methanol and stained with haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS). Paraffin sections from the primary tumour and xenografts were then stained with the same reagents.

For immunocytochemistry the antibodies used included monoclonal antibodies to desmin (1:50; Dako, Kyoto, Japan), and polyclonal antibodies to myoglobin (1:1000; Dako). The cells grown in chamber slides were washed in PBS and fixed in cold acetone for 5 min. The cells were reacted with each of the primary antibodies for 1 h at room temperature. The attached antibodies were then visualized by an avidin-biotin-peroxidase complex (ABC) procedure (Vector Laboratories, Burlingame, Calif.). The paraffin sections from the original tumours and the xenografts were also examined using the same procedure.

At passage 2 the cells were analysed by the standard trypsin–Giemsa banding techniques. They were incubated in DMEM/F-12 medium containing colcemid (0.05 μg/ml) and 10% FCS at 37°C for 24 h, treated with 0.075 M KCl for 10 min, then fixed in Carnoy's solution, dropped on a slide glass, and dried at 37°C. Samples were treated with 0.1% trypsin at 27°C for about 10 s and stained with Giemsa for 10 min. The karyotypes were made according to the International System for Human Cytogenetic Nomenclature [43].

Cultured cells at passage 7 of FU-MMT-3 were used for heterotransplantation. The cells (5×10^7 cells suspended in 0.3 ml of PBS) were injected into the subcutis of 4- to 6-week-old female nude mice (BALB/c nu/nu, Nippon Kurea, Tokyo, Japan). The mice were maintained in a pathogen-free environment.

High-molecular-weight cellular DNA was extracted from three MMMT cell lines by standard protocols, as previously described [15]. The DNA (10 μg each) was digested with a restriction enzyme EcoRI, subjected to electrophoresis through agarose gel, and transferred to nitrocellulose membrane filters as reported by Southern [40]. The filters were hybridized with a ^{32}P-radiolabelled DNA probe. The probe used consisted of a third exon of c-*myc* and MYCN (Oncor, Gaithersburg, Md.).

Results

From the beginning of the primary cultures, FU-MMT-3 showed excellent growth without contact inhibition. Because a small number of fibroblasts were found to contaminate the early cultures, we maintained the cultures without transferring them to subcultures until the tumour cells had grown confluent. The fibroblasts disappeared gradually from the cultures without selective fibroblastic removal methods. Confluent tumour cell layers were thus obtained within 2 months of the primary cultures, and their subcultures were made every 1 or 2 weeks at 1:2 or 1:4 dilution. The average doubling time at passage 5 was 29 h. FU-MMT-3 cells were in continuous culture up to the 57th passage over 2 years.

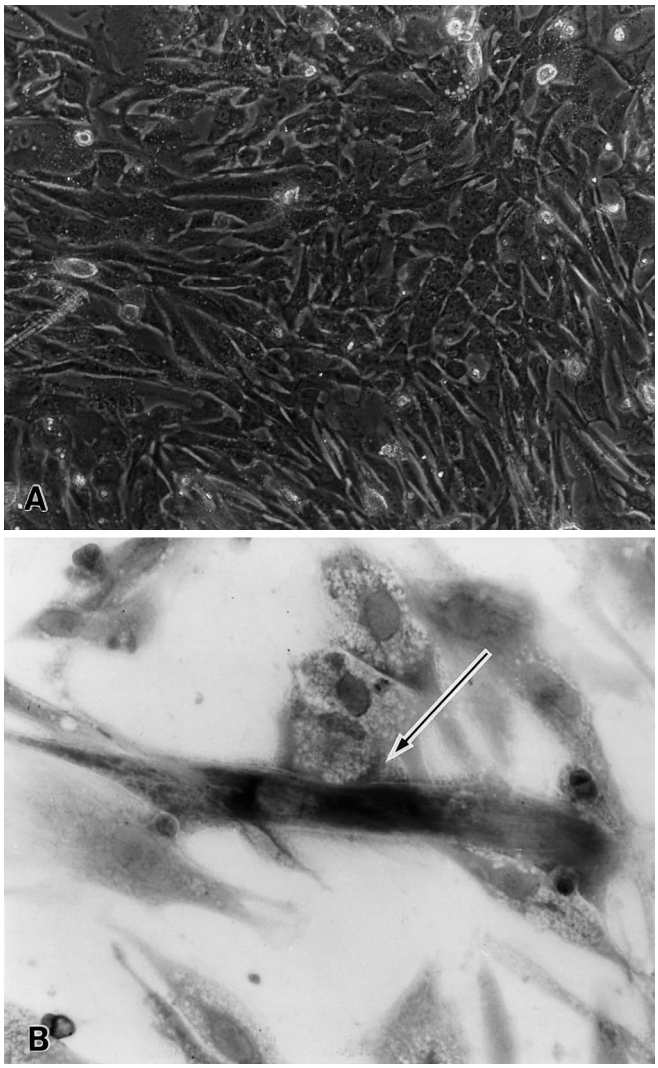

Fig. 2A, B Morphological and histochemical features of FU-MMT-3. (**A**) FU-MMT-3 cells showing a mixture of strap-like cells and round to polygonal cells in vitro (Phase contrast, ×320). (**B**) Periodic acid-Schiff stain demonstrating abundant glycogen in FU-MMT-3 cell (*arrow*)(×380)

Inoculation of FU-MMT-3 cells into the subcutis of athymic nude mice resulted in the development of palpable tumour nodules. FU-MMT-3 xenografts grew rapidly, exceeding 20 mm in diameter within 1 month of inoculation. The FU-MMT-3 cell line was followed for more than 5 heterotransplantations in series.

Microscopically, every FU-MMT-3 culture was composed exclusively of sarcomatous cells, including spindle cells and round to polygonal cells (Fig. 2A). These cells grew haphazardly in multiple layers and occasionally formed interlacing bundles. By PAS stain with or with-

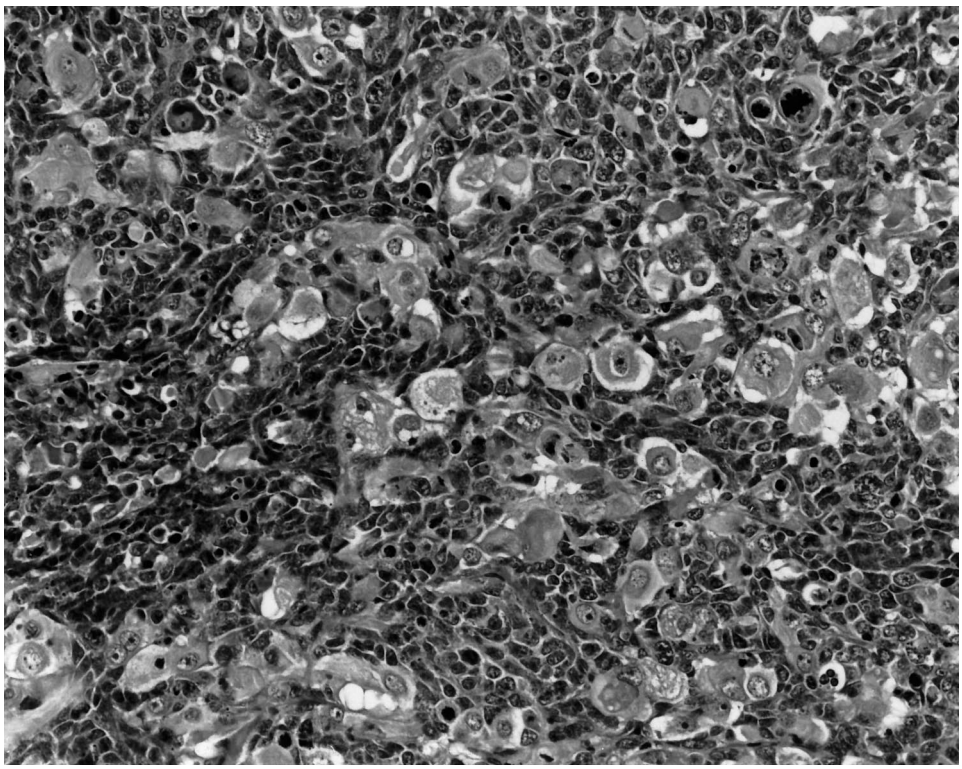

Fig. 3 Histology of FU-MMT-3 xenograft shows rhabdomyoblastic differentiation, resembling the original tumour (H & E, ×190)

Table 1 Immunophenotypes of FU-MMT-3, the original tumour, and the xenograft (++ >50% positive cells, + <50% positive cells, – negative reaction)

	FU-MMT-3 (sarcoma)	Original tumour (carcinoma/sarcoma)	Xenograft (sarcoma)
Desmin	++	–/++	++
Myoglobin	+	–/+	+

out prior diastase digestion, abundant glycogen was demonstrated in many FU-MMT-3 cells (Fig. 2B). However, they lacked distinct cross-striations in the cytoplasm, and thus resembled the findings in the original tumour.

The xenografts of FU-MMT-3 consisted of sarcomatous tissue resembling the RMS in their original tumour, but no epithelial (carcinomatous) components were present (Fig. 3).

The immunocytochemical reactivities in FU-MMT-3, the original tumour, and the xenograft are shown in Table 1. The cultured cells of FU-MMT-3 expressed the myogenic antigens (desmin and myoglobin) (Fig. 4A, B). The reactivities in the original tumour and the xenograft (Fig. 4C) were similar to those in the cell line.

Eight cells of an FU-MMT-3 cell line at passage 2 were karyotyped. The modal chromosome number was 74–80, and analysis of G-banding showed complex aberrations. The clonal abnormalities common to the eight FU-MMT-3 cells we karyotyped were: XX, +1, +1, +del(1)(q12), +3, +3, +add (3)(p11), +4, +5, +6, +8, +del(11)(p13), +12, +add(13)(p13), +18, +20, +21, +22, +5–14 mar [cp8] (Fig. 5). Although the extra copies of chromosome 8 were common to both MMMT cell lines previously reported, no other specific abnormalities were found in these three cell lines.

Cellular DNAs from three MMMT cell lines consisting of FU-MMT-3, FU-MMT-1, and FU-MMT-2 were examined, and all three showed an amplification of the c-*myc* gene, while none demonstrated MYCN gene amplification. All of these samples exhibited similar levels of amplification of c-*myc* gene (Fig. 6A). Comparisons of the c-*myc* DNA bands with cultured human placental DNA were performed by the serial dilution method. The c-*myc* gene was amplified approximately fourfold to eightfold in each sample (Fig. 6B).

Discussion

Recently, several tissue culture studies in uterine MMMT have been reported [14, 15, 21, 35]. In our previous studies, two uterine MMMT cell lines and the subclones were established and their biological characteristics were examined in vitro and in vivo [14, 15]. Our findings supported the theory of a single stem cell origin of MMMT and substantiated the current concept of this neoplasm as a “metaplastic carcinoma”. In this study, we examined the biological characteristics of a newly established MMMT cell line (FU-MMT-3), the third “pure” RMS cell line derived from heterologous MMMTs [14,

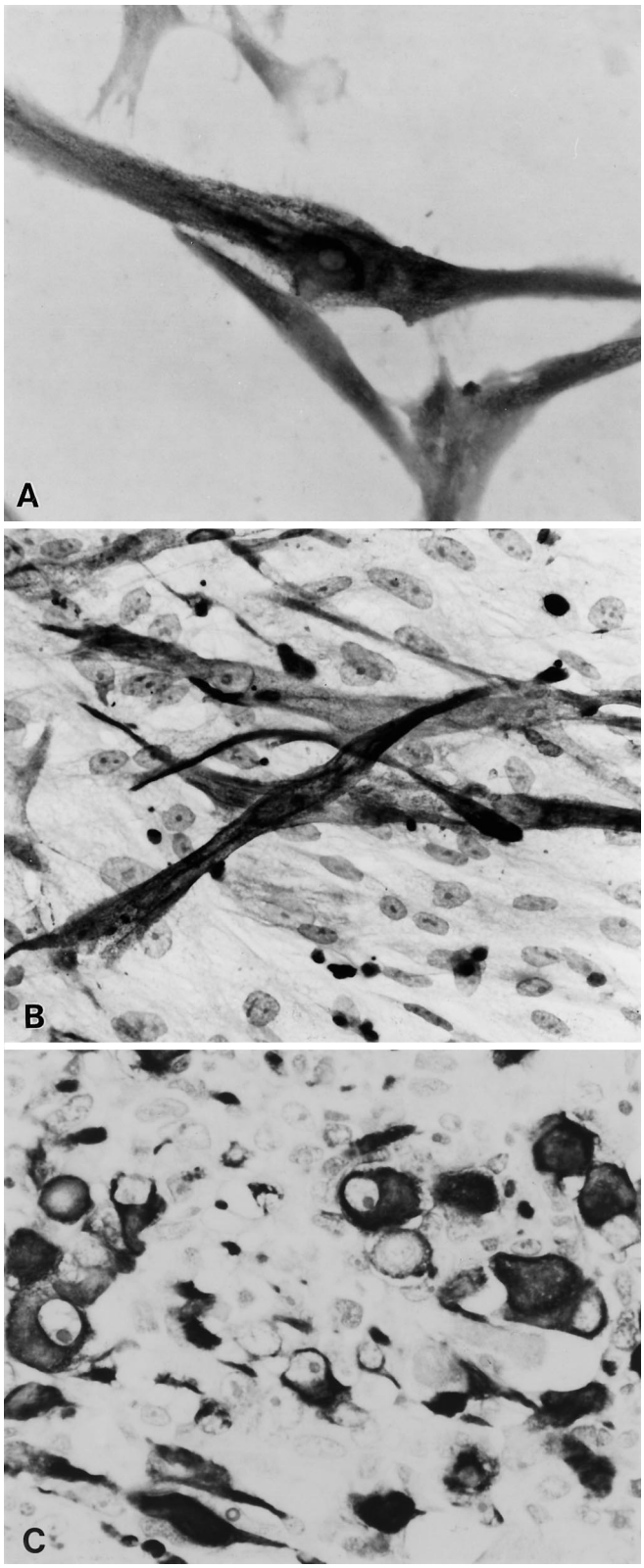

Fig. 4A–C Immunocytochemical features of FU-MMT-3 and the xenotransplanted tumour. (**A**) The FU-MMT-3 cells were stained positive for myoglobin (ABC technique, ×410). (**B**) Desmin was strongly demonstrated in FU-MMT-3 cells (ABC technique, ×370). (**C**) The FU-MMT-3 xenograft were also stained positive for desmin (ABC technique, ×380)

35]. We also evaluated whether consistent cytogenetic or molecular alterations existed in our three RMS cell lines.

Many cytogenetic studies of RMS in soft tissue have been made, and a specific characteristic alteration of these tumours has been found; translocation (2;13)(q37;q14) is present in most alveolar RMS [12]. Several studies have also detected cytogenetic alterations in MMMTs [14, 27, 30, 32]. Most showed a complex series of chromosomal changes, suggesting marked genomic instability of these tumours. Milatovich et al. analysed six homologous endometrial MMMTs, three of which had abnormal karyotypes including structural abnormalities of chromosomes 1, 3, and 5 [30]. In addition, Laxman et al. reported that three of four MMMTs showed a common abnormality of materials deleted from the long arm of chromosome 11 distal to band 11q22 [27]. They also suggested that genomic alterations in the region 11q22 may be specific for malignant smooth muscle tumours of the uterus. However, no consistent karyotypic changes have yet been observed in any of the MMMTs reported. Moreover, only a few reports deal with cytogenetic examination of heterologous MMMT [14, 32]. In our previous study, both MMMT cell lines with rhabdomyoblastic features showed extra copies of chromosome 8; FU-MMT-1 had trisomy of chromosome 8 and FU-MMT-2 also had $+8p^{-}$ [14]. In addition, a newly established MMMT cell line, FU-MMT-3, which is compatible with the features of RMS, also demonstrated trisomy of chromosome 8 in all karyotyped cells. Trisomy of chromosome 8 was demonstrated in the two “pure” RMS cell lines (FU-MMT-1 and FU-MMT-3). Nilbert also reported a case of uterine MMMT with a component of RMS that showed abnormal karyotypes including quadsomy of chromosome 8 [32], and no reports have demonstrated extra copies of chromosome 8 in MMMTs without rhabdomyoblastic differentiation, or in endometrial stromal sarcomas [23]. This suggests that there may be some association between rhabdomyoblastic differentiation and extra copies of chromosome 8 in MMMT.

In the case of botryoid RMS occurring in the uterine cervix or vagina there are few reports on examinations of the cytogenetic aberrations, except for a unique case of one in the uterine cervix of a 19-year-old woman [36]. The cytogenetic abnormality showed trisomies of chromosomes 13 and 18 and an extra copy of chromosome 1, without chromosome 8 alterations. We thus consider that there might be some pathogenetic differences between RMS in MMMT and pure botryoid RMS in the female genital tract. However, further cytogenetic and molecular analyses in these neoplasms are called for.

In our studies, three RMS cell lines have been established from three patients with heterologous MMMTs. Although we have also tried to establish cell lines from three cases of homologous MMMT in the same period, we have not been successful in this, which suggests a more aggressive biological behaviour of rhabdomyoblasts than of the homologous mesenchymal elements in MMMTs. This behaviour might also be reflected in the short doubling times of the cultured cells; 26 h for FU-

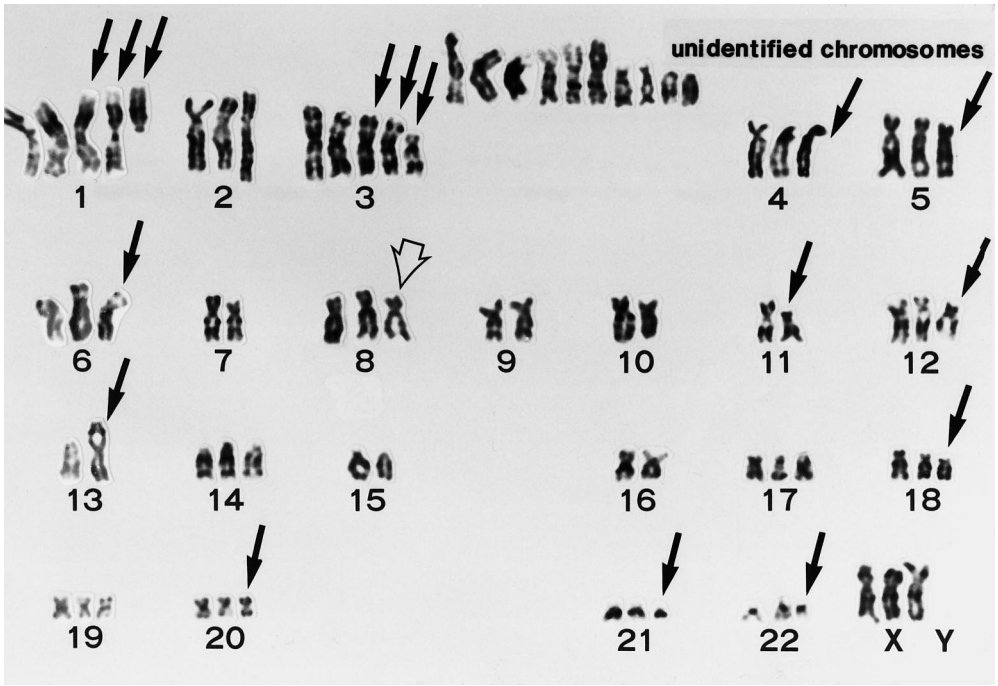

Fig. 5 Giemsa-banded karyotypes of FU-MMT-3. The clonal abnormalities common to FU-MMT-3 cells were: XX, +1, +1, +del(1)(q12), +3, +3, +add (3)(p11), +4, +5, +6, +8, +del(11)(p13), +12, +add(13)(p13), +18, +20, +21, +22, +5–14 mar [cp8]. *Arrows* show the clonal abnormalities and *white arrow* indicates trisomy of chromosome 8

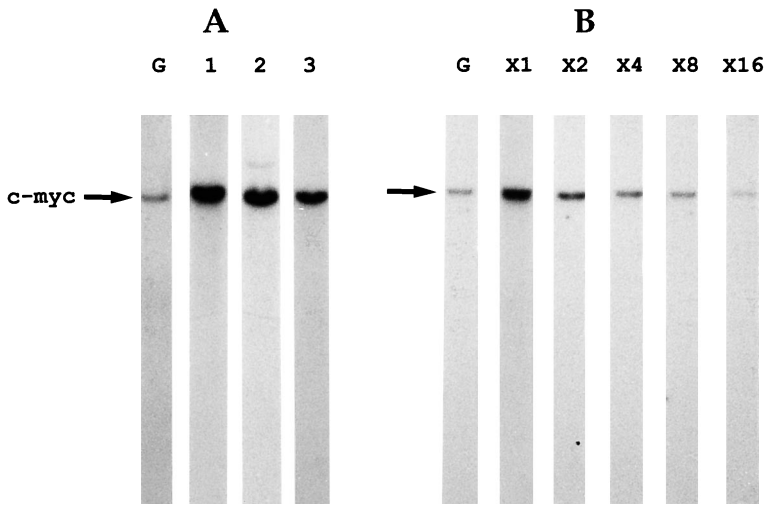

Fig. 6A, B Southern blot analysis for c-*myc* in FU-MMT-1, FU-MMT-2, and FU-MMT-3 (*G*: *Germ line*, *Lane 1*: *FU-MMT-1*, *Lane 2*: *FU-MMT-2*, *Lane 3*: *FU-MMT-3*). c-*myc* fragment length after digestion of human DNA with Eco RI: 13 kb. (**A**) All of these cell lines showed very similar levels of c-*myc* gene amplification. (**B**) Comparison of the c-*myc* DNA bands in FU-MMT-3 with cultured human placental DNA were performed by the serial dilution method. The c-*myc* gene was amplified by approximately fourfold to eightfold in each sample

MMT-1, 30 h for FU-MMT-2, and 29 h for FU-MMT-3. Some reports in the clinicopathological studies of MMMT described the more aggressive clinical behaviour of MMMTs with RMS than of homologous MMMTs [33]. Even in our cases, the prognoses of the three patients affected with MMMT with rhabdomyoblastic differentiation were poorer than those of the patients with homologous MMMTs. All of the patients died of rapid tumour progression within 3 years after their first surgery. However, the exact nature of the high aggressivity of RMS in MMMT is still unclear, so that further biological studies are considered necessary.

Recently, many molecular biological studies of gynaecological malignancies have been carried out. In the studies of endometrial carcinoma, several reports showed molecular alterations of some oncogenes, including c-*erb*B-2, K-*ras*, c-*fms*, c-*myc* [4, 7, 17, 24, 31]. However, few studies have been concentrated on the alterations of oncogenes in uterine MMMT, except for our own previous study, which showed the amplification of the c-*myc* gene, which is recognized as a dominant-acting oncogene but encodes a 62-kDa transcriptional factor thought to regulate the cell cycle transition from G_0 to G_1 [2], in MMMT cells with rhabdomyoblastic features in vitro and in vivo [15]. Therefore, the current study also examined the presence or absence of c-*myc* gene amplification in two pure RMS cell lines derived from uterine MMMTs by a Southern blot analysis, and as a result, a high frequency of c-*myc* gene amplification was demonstrated. In studies of endometrial carcinomas a low frequency of amplification for this gene is usually found [17, 24, 31]. In our current study, we observed evidence

that a high frequency of c-*myc* gene amplification exists in MMMTs, which suggests oncogenic differences between MMMTs and ordinary endometrial carcinomas and a different nature of these two tumours of endometrial origin. In addition, c-*myc* gene amplification might also be associated with the pathogenesis of rhabdomyoblastic differentiation in MMMT. Further analyses using molecular biology are needed in both heterologous MMMTs and homologous MMMTs.

The MYCN oncogene, previously known as N-*myc*, is a member of the MYC gene family, which encodes proteins that play a part in the control of cell proliferation and differentiation. The amplification of MYCN gene has been detected in several malignant neoplasms, including neuroblastoma [5], retinoblastoma [42], medulloblastoma [19], astrocytoma [18], and small cell carcinoma of the lung [45]. As far as we know, our current study is the first to examine this gene alteration in MMMT. In the studies of RMS the amplification of the c-*myc* gene is less common, while MYCN gene amplification is frequently shown in alveolar RMS [8, 9, 13]. Such evidence suggests the existence of biological differences between RMS in soft tissue and that in MMMTs.

Missense mutations and rearrangement of the *p53* gene on chromosome 17p13 are among the most common genetic alterations observed in human malignancies [22]. Such alterations have also been found in childhood RMS and in some gynaecological malignancies, including uterine sarcomas [16, 26, 28]. Liu et al. reported that overexpression of *p53* occurred in 17 of 22 (77%) cases of heterologous MMMT and 9 of 19 (47%) cases of homologous MMMT, thus suggesting differences between heterologous and homologous MMMT [28].

FU-MMT-3 is considered to provide a useful system for the study of the biological behaviour of RMS in MMMTs. Our findings suggest that c-*myc* gene amplification and extra copies of chromosome 8 may be associated with rhabdomyoblastic differentiation in MMMT.

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