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Establishment and characterization of a new cell line from human bladder cancer (JMSU1)

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Abstract A new human bladder cancer cell line designated JMSU1 has been established from malignant ascitic fluid of a 75-year-old Japanese man with bladder cancer, and maintained in culture for more than 7 years and over 240 passages. Inverted phase-contrast microscopy revealed that JMSU1 was composed of morphologically distinct cells (polygonal to spindle-shaped cells), showing morphological heterogeneity in vitro. Histological examination of xenografts showed poorly differentiated transitional cell carcinoma, resembling the original tumor. Immunohistochemical staining for cytokeratin and electron microscopic examination suggested that JMSU1 was of epithelial origin. Chromosome analysis gave a modal number of 69 with no Y chromosome. Isozyme analysis (LDH, G6PD, and NP) showed the mobility pattern of human type B. DNA fingerprint analysis demonstrated that there was no cross-culture contamination of JMSU1 during the passages. In conclusion, a newly established and well-characterized cell line, JMSU1, offers promising material for the investigation of the biological properties of bladder cancer.

Key words Bladder cancer · Cell line · JMSU1 · Heterogeneity · DNA fignerprint

Attempts to establish long-term cultured cell lines of human bladder cancer have been made and many human bladder cancer cell lines have been established from primary or metastatic sites of bladder cancer [2, 7, 15, 16, 19]. These cell lines have been used to investigate sensitivities to anticancer drugs, karyotypes, expression of tumor antigens or oncogenes, and growth factor production of bladder cancer. For some cell lines, however, neither confirmatory clinical data nor characterization data are available, thereby creating doubts as to the origin and individuality of the cell lines. Nelson-Rees et al. reported that long-term cultured cell lines sometimes have cross-culture contamination by the same or other species during manipulations [10]. Furthermore, sublines of a previously established cell line are sometimes reported as the independent cell line. For example, MGHU1, MGHU2, and EJ1, previously thought to be independent bladder cancer cell lines, were demonstrated to be sublines of the previously established cell line T24 [8, 12]. This evidence indicates that extensive characterization of cell lines is needed to give the experimental results validity and reproducibility for cancer research. With this aim, we tried to establish a new human bladder cancer cell line in our laboratory. This study describes the establishment and extensive characterization, especially in terms of individuality, of a new cell line derived from malignant ascitic fluid of a Japanese man with bladder cancer.

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Materials and methods

Patient details

A 75-year-old Japanese man with bladder cancer underwent palliative cystectomy with urinary diversion on 22 March 1988. Malignant ascitic fluid was observed after the operation, and 20 ml ascitic fluid was collected for cell culture on 13 April 1988. The patient received neither chemotherapy nor radiation therapy and died on 16 April 1988. Autopsy and surgical material showed that poorly differentiated transitional cell carcinoma (grade 3) of the bladder had metastasized to lungs, bone marrow, liver, lymph nodes, and the

intraperitoneal space (pT4N2M1), and that there were no other malignancies except for the bladder cancer.

Cell culture

Twenty milliliters ascitic fluid was centrifuged, and the pellet was washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The cell suspension was placed in 25-cm^2 tissue culture flasks (Costar, Cambridge, Mass., USA) and incubated in a humidified 5% CO_2 atmosphere at 37 °C. Medium was changed twice a week. The cells at preconfluence were briefly exposed to trypsin-ethylendiaminetetraacetic acid (EDTA) solution, washed with PBS, and then subcultured at a split ratio of 1:10.

Growth curves

Single-cell suspension of log-phase cells was seeded into six-well tissue culture plates (Costar, Cambridge, Mass., USA) at 1×10^5 cells/well in triplicate. The cells were detached with trypsin-EDTA solution, stained with erythrosin, and then counted with a hemocytometer on days 0, 2, 4, 6, and 8.

Colony formation in soft agarose

Anchorage-independent growth was examined by colony formation in soft agarose. 1×10^5 cells in 0.5 ml 0.3% agarose were seeded over a 0.5% agarose base in 35-mm petri dishes. After 12 days in culture, colony numbers (>50 μm in diameter) were counted using the inverted phase-contrast microscope.

Cell storage

Cells on 25-cm^2 tissue culture flasks were trypsinized, washed with PBS, and then resuspended in RPMI 1640 supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO). The cell suspension was aliquoted in 2-ml freezing vials. Vials were kept at 4 °C for 1 h and then transferred in a −80 °C freezer. Some of the vials were further placed in a liquid nitrogen (LN_2) freezer.

Tumorigenicity in athymic nude mice

Five-week-old male Balb/c nu/nu mice were purchased from Doken (Ibaragi, Japan) and used for in vivo experiments. Mice were given an s.c. injection of 5×10^6 viable cells and kept under specific pathogen-free conditions. When palpable nodules were observed, they were surgically removed and prepared for morphological study.

Light microscopy

Surgically removed s.c. tumors established in athymic nude mice were fixed in 10% buffered formalin, stained with hematoxylin and eosin, and examined by light microscopy. Immunohistochemical staining for keratin and vimentin was performed by the method reported previously [9].

Electron microscopy

Subcutaneous nodules grown in athymic nude mice were cut into small pieces. They were fixed with 2.5% glutaraldehyde in 0.005 M cacodylate buffer, postfixed in 1.5% osmium tetroxide, and then processed by standard methods for analysis under the electron microscope.

Chromosome analysis

Chromosome analysis was performed by the G-banding method [18]. Briefly, monolayer cells were cultured with Colcemid (Sigma, Mc., USA) for 4 h, then exposed to 0.075 M KCl solution for 10 min at room temperature. The cells were fixed with methanol/acetic acid (3/l, v/v), dropped onto clean slides, and air dried at 37 °C. After treatment with 0.25% trypsin solution for 3 min, the slides were rinsed with PBS, air-dried, and then stained with Giemsa solution.

Isozyme analysis

Isozyme phenotypes of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD), and purine nucleoside phosphorylase (NP) were analyzed with the Authentikit system (Innovative Chemistry, Marshifield, Mass., USA). Briefly, the cells were detached with trypsin-EDTA solution, washed with PBS, and lysed by freeze-thaw in 0.9% NaCl solution including 1.0% Triton-X 100. The cell extracts were then centrifuged and supernatants were frozen until use. After electrophoresis in agarose gel, isozyme phenotype was determined by comparison of migration distances according to the instructions attached to the kit. The extracts of HeLa S3 (human cervical carcinoma) and L929 (murine cell line derived from connective tissue) were used as reference lines.

DNA fingerprint analysis

DNA fingerprint analysis was performed by the protocol reported previously [5]. The bladder cancer cells including JMSU1, MGHU1 [7], and T24 [2] obtained from the Japanese Cancer Research Resources Bank (JCRB) were detached with trypsin-EDTA solution and washed with PBS, and the dry pellets were then stored at −80 °C until use. Genomic DNA was isolated by standard protocols and digested with restriction endonuclease *Hin*fI (TOYOBO, Tokyo). The digested DNA samples were electrophoresed in 1.0% agarose gel in TAE. The gel was blotted onto a nylon membrane filter and air dried at room temperature. The filter was hybridized with ^{32}P-labeled DNA probe "Core," washed, and then exposed to X-ray film with intensifying screens at −80 °C.

Results

Establishment of cell line

The primary culture of JMSU1 attained a monolayer within 1 week and was subcultured at a split ratio of 1:1. Subsequently, cells were subcultured at greater split ratios and increased time intervals. The mean doubling time of JMSU1 was 32 h at the 75th passage. JMSU1 is presently in its 240th passage after 7 years in culture. As shown in Fig. 1, JMSU1 grows in typical epithelial cobblestone patterns without contact inhibition. However, JMSU1 in vitro was composed of morphologically

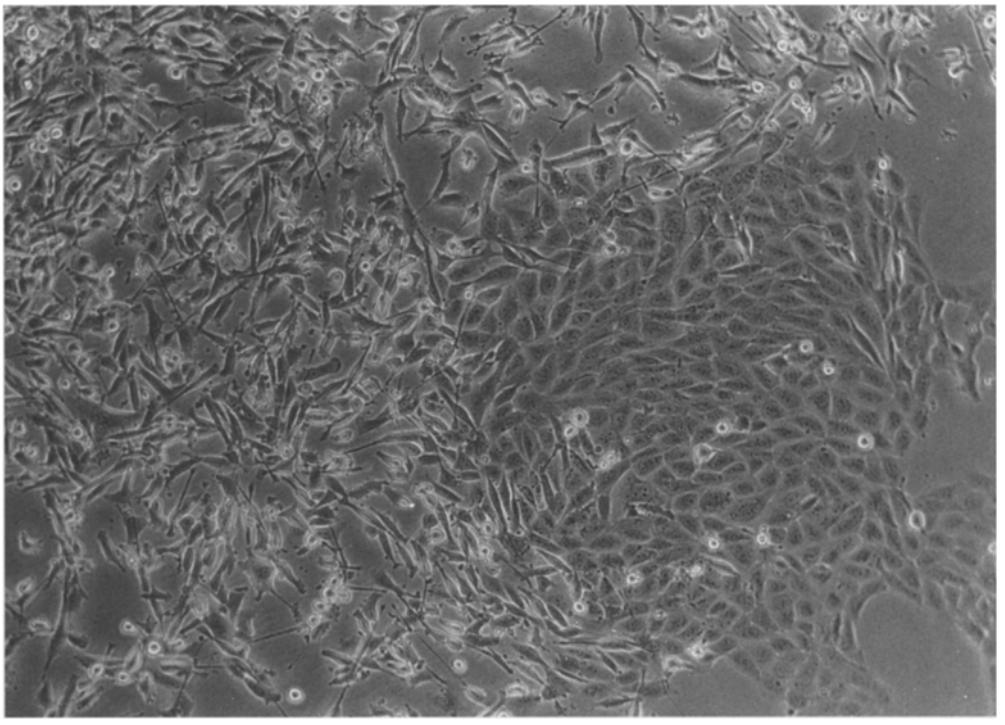


Fig. 1 Inverted phase-contrast micrograph of JMSU1

distinct cells: polygonal to spindle-shaped cells. This morphological heterogeneity in vitro has been observed over 240 passages. We have already obtained several clones of JMSU1 with distinct morphological appearances in vitro, and clonal analysis showed that morphologically distinct cells were derived from human bladder cancer (data not shown).

Liquid nitrogen storage

When revived from liquid N_2 storage, JMSU1 attached readily and had a morphological appearance similar to the cells in continuous culture.

Tumorigenicity

Tumorigenicity was examined by the growth in soft agarose and zenotransplantation in athymic nude mice. JMSU1 showed anchorage-independent growth, JMSU1 at the 75th passage yielded a plating efficiency of 3.6% with 344 ± 94 colonies/well (mean $\pm$ SD). Subcutaneous tumor was observed in five out of five mice examined 2 weeks after inoculation. Mice were put to death and examined for metastasis 4 weeks after inoculation. Metastasis was not found in lymph nodes, lungs, or liver.

Morphology

Histological examination of subcutaneous tumors established in athymic nude mice revealed that they were poorly differentiated transitional cell carcinomas resembling the original tumor (Fig. 2). There was no significant histological heterogeneity in either surgical specimens or xenografts stained with H&E. Immunohistochemical studies showed that JMSU1 was positive for keratin and vimentin. Electron microscopy showed the desmosomes and intracytoplasmic tonofibrils (Fig. 3). There was no evidence of intracytoplasmic viral particles or cell surface budding.

Karyotype

More than 50 metaphases of JMSU1 were examined. Chromosome numbers varied between 66 and 74 with the modal number of 69 at the 12th passage (Fig. 4). There were 11 marker chromosomes (Fig. 5). No Y chromosome was observed although it was expected in the male-derived cells.

Isozyme analysis

Mobility patterns of isozymes including LDH, G6PD, and NP were examined (Fig. 6). The isozyme phenotype of JMSU1 was different from that in L929 (murine cell line) and in HeLa S3 (human type A) and identical with the human type B.

DNA fingerprints

Elimination of cross-culture contamination was needed since two bladder cancer cell lines including MGHU1 and T24 were maintained in our laboratory. We examined the DNA fingerprint patterns of Southern blots of genomic DNAs isolated from the frozen stocks of

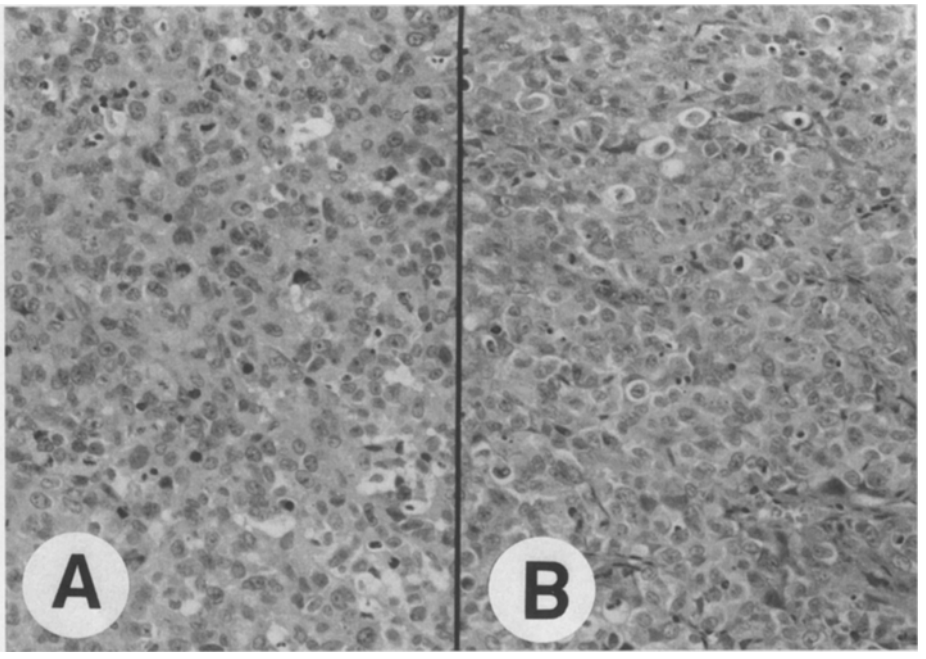


Fig. 2A, B Histological features of original tumor and xenograft of JMSU1. **A** Original tumor, **B** xenograft in athymic nude mice. H&E, × 400

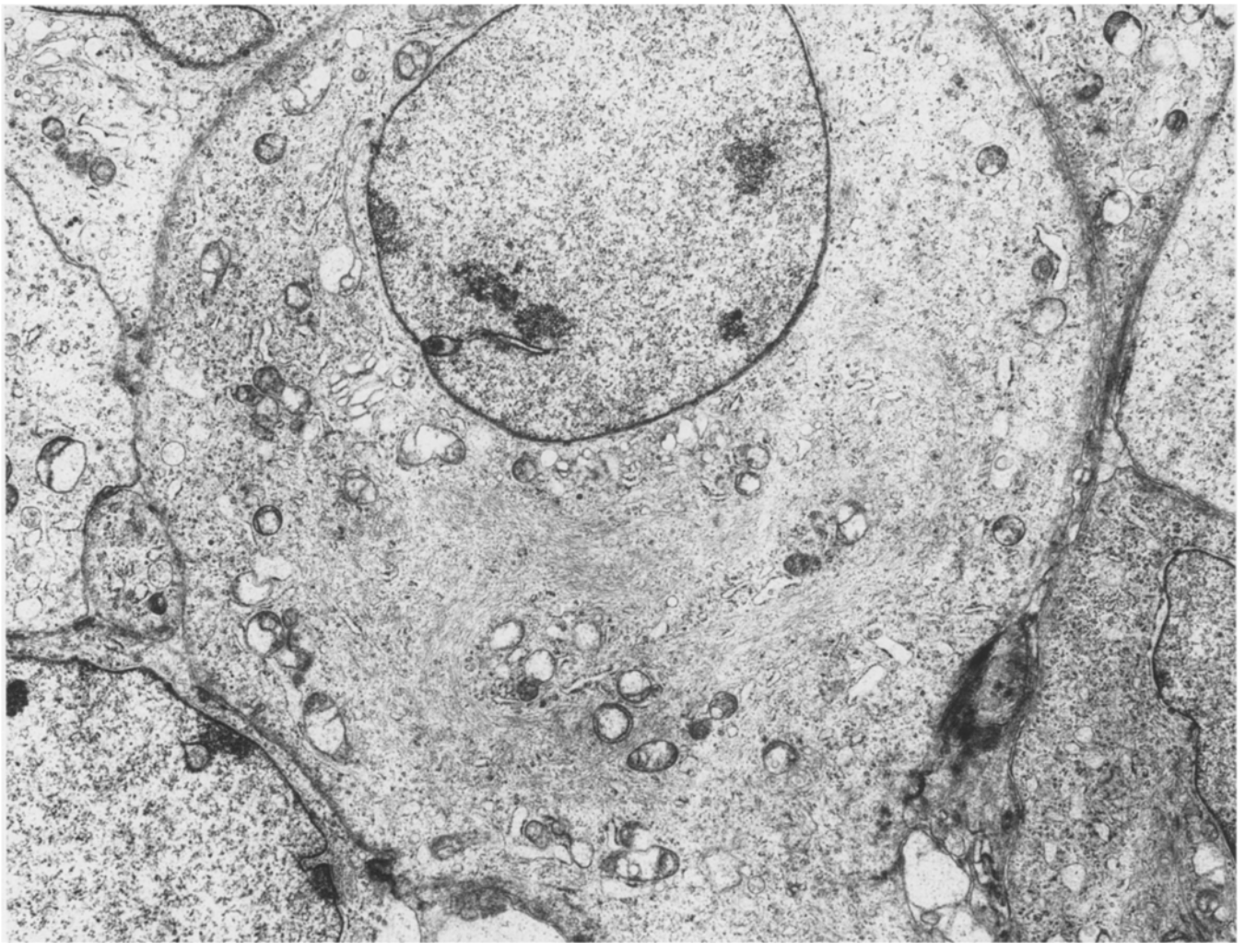


Fig. 3 Electron micrograph of JMSU1, × 5000

original cells without culture, JMUS1 at the 5th and 240th passages, MGHU1, and T24. As shown in Fig. 7, DNA fingerprint patterns of JMSU1 at the 5th and 240th passages were the same as the pattern of the original cells without culture but different from those of MGHU1 and T24, indicating that JMSU1 with morphological heterogeneity in vitro was not contaminated. On the other hand, MGHU1 had an identical DNA fingerprint pattern to that of T24, suggesting that MGHU1 was derived from T24, as pointed out previously [8, 12].

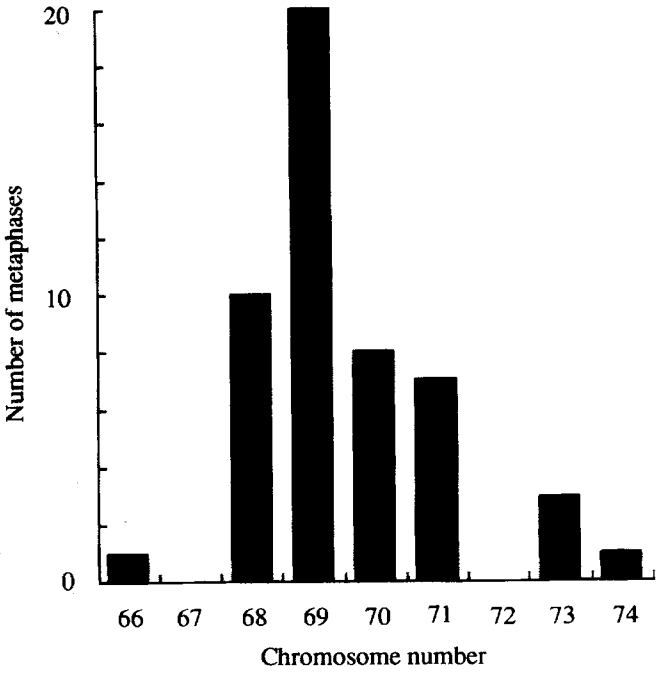


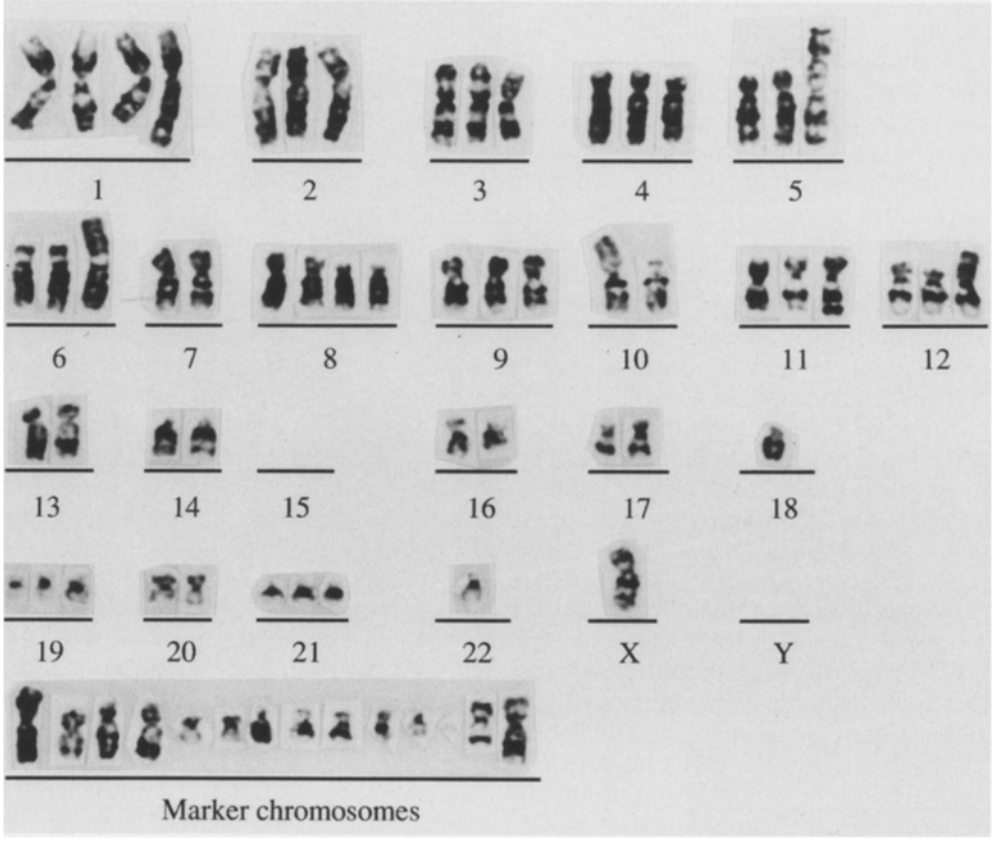
Fig. 4 Distribution of chromosome number at the 72th passage

Discussion

A new cell line established in our laboratory has been in culture for more than 7 years and more than 240 passages. The successful establishment of a new cell line was partly due to the source of material, because effusions have the advantage of providing large numbers of dissociated viable tumor cells with little or no contamination by fibroblasts.

In the present study, we characterized JMSU1 derived from malignant ascitic fluid of a male patient with bladder cancer. The tumorigenicity was demonstrated by the growth in soft agarose and xenografts in athymic nude mice. The epithelial nature of JMSU1 was established by the following findings: (a) cobblestone appearance without contact inhibition in vitro, (b) positive staining for keratin on immunohistochemical study, and (c) presence of desmosomes shown by electron microscopy. Furthermore, JMSU1 was of human bladder origin according to the following criteria: (a) inter- or intraspecies specificity, demonstrated by human karyotype,

Fig. 5 Karyotype of JMSU1 at the 72th passage by G-banding method



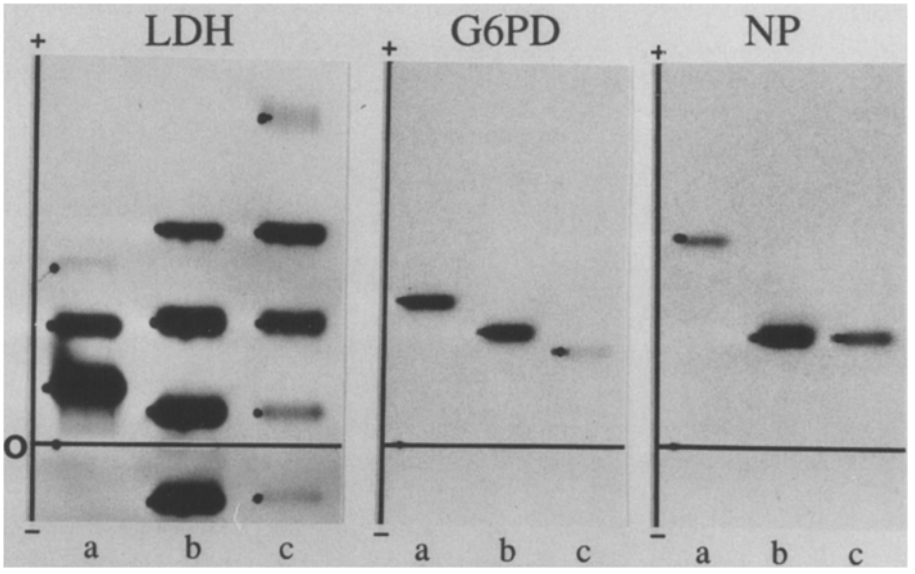


Fig. 6 Zymographs of LDH, G6PD, and NP. *Closed circle* was placed on left side of each band to measure the distance from origin to each band. *a* L929, *b* HeLa S3, *c* JMSU1 at the 72th passage, *O* origin

isozyme phenotype, and DNA fingerprint analysis; and (b) organ specificity, supported by morphological evidence of an epithelial structure, histological similarity to the original tumor (transitional cell carcinoma, grade 3), and autopsy findings that there were no other malignancies except for bladder cancer. Although the source of JMSU1 was not a primary lesion but ascitic fluid, this evidence indicates that JMSU1 is a newly established cell line originating from human bladder cancer.

Chromosome analysis demonstrated the absence of Y chromosome in JMSU1, although it was expected in the male-derived cells. Peterson et al. [13] examined Y chromosome in many cell lines and reported that cell lines often lose Y chromosome even though they were derived from male subjects [13]. The exact mechanism is still unclear but rearrangements including the loss of Y chromosome may occur in vivo before initiation of the culture in vitro. In the case of JMSU1, the loss of Y chromosome might occur not during the in vitro culture but during the progression steps of bladder cancer in vivo, because the chromosome analysis of original cells (cancer cells in ascitic fluid) showed that they had already lost Y chromosome (data not shown).

For identification of the cell line, there are several techniques including immunological, chromosomal, and enzymological methods [11,14]; the latter two were employed in the present study. Although these methods can characterize the cell line species, sex, and racial species, it is very difficult to distinguish among cell lines derived from the same species. DNA fingerprint analysis developed by Jeffreys et al. [6] uses a polymorphic DNA marker as a probe to recognize DNA polymorphism with Southern blot analysis. This technique can identify one individual unequivocally and is used to detect cross-culture contamination [5,17]. Furthermore, DNA fingerprint pattern is demonstrated to be stable throughout many continuous passages [5]. In the present study, JMSU1, which had morphological heterogeneity in vitro, had an identical DNA fingerprint pattern over 240 passages, indicating that JMSU1 has no cross-culture contamination. On the other hand, as described in the "Introduction", MGHU1 was previously found to be derived from T24 as shown by the enzymological method [8,12]. The present study with DNA fingerprint analysis clearly demonstrated that these two cell lines had the same DNA fingerprint pattern, supporting the conclusion that their origin is the same. Accordingly, DNA fingerprint analysis is a good tool with which to examine the identity of each cell line, and periodical monitoring of DNA fingerprint is recommended to detect accidental cross-culture contamination.

Intratumoral heterogeneity has been demonstrated in a variety of tumors [4]. Bladder cancer has been also demonstrated to be a heterogeneous population of cells with respect to cystoscopic appearance, histology, karyotype, DNA contents, cellular surface antigens, and chemosensitivity [1,3]. In the present study, JMSU1 demonstrated morphological heterogeneity when viewed with the inverted phase-contrast microscope although there was evidence that long-term cultured cell lines in vitro can lead to phenotypic instability such as morphological changes. It seems that the morphological heterogeneity of JMSU1 already exists in vivo rather than the morphological changes taking place in vitro, since it was observed at the early stages of passage in culture. This finding suggests that JMSU1 consists of clones with distinct biological properties.

In order to elucidate whether JMSU1 has histological heterogeneity or not, subcutaneous tumors established in athymic nude mice were examined for histological features of JMSU1. Although JMSU1 showed morphological heterogeneity in vitro, there was no significant histological heterogeneity in surgical specimens and xenografts stained with H&E. It is not clear why the discrepancy between morphological heterogeneity

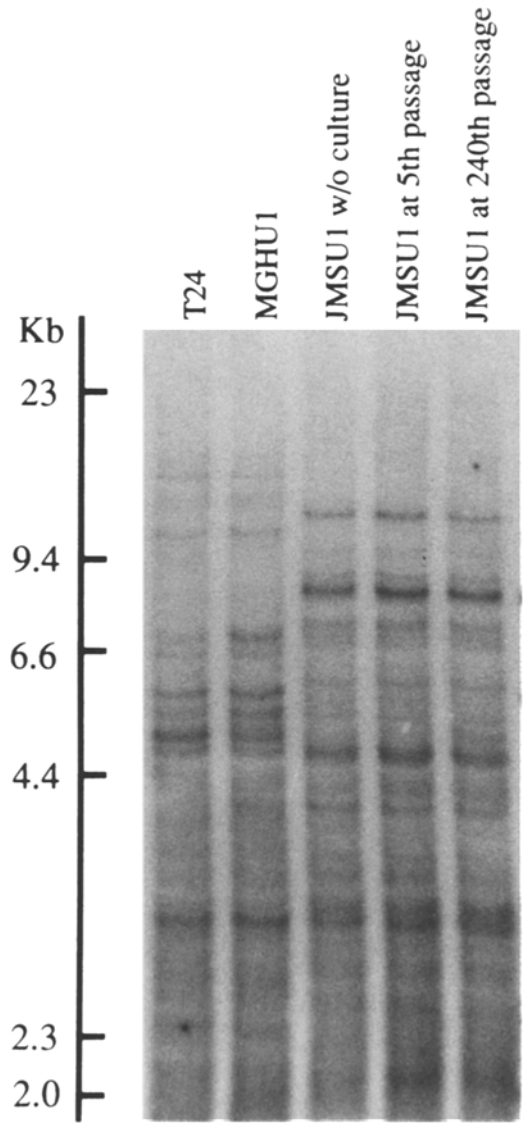


Fig. 7 DNA fingerprints of Southern blots of genomic DNAs from bladder cancer cell lines. JMSU1 w/o culture, the uncultured cells aspirated from ascitic fluid

in vitro and in vivo was seen. Clones which have almost the same histological features but distinct morphological appearances in vitro might exist in JMSU1, which possibly results in there being no significant histological heterogeneity in xenografts stained with H&E. Alternatively, there might be clones with distinct histological features which have distinct tumorigenicity in athymic nude mice. Zenografts would be mainly composed of clones with high tumorigenicity, resulting in no significant histological heterogeneity. Further analysis of JMSU1 at the clone level is needed to elucidate whether JMSU1 possesses heterogeneity in its biological properties, including histological features, tumorigenicity, and oncogenes or tumor suppressor gene expression. In our laboratory, we have already obtained several clones of JMSU1 with distinct morphological appearances in vitro, and clonal analysis is now in progress.

In conclusion, the present study demonstrated that the established cell line designated JMSU1 is a new human bladder cancer cell line with morphological heterogeneity in vitro. JMSU1 is a readily available and reliable source of material for the study of the biological properties of human bladder cancer, especially the heterogeneity of bladder cancer.

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