Establishment and characterization of a new cell line, FRTK-1, derived from human malignant rhabdoid tumor of the kidney, with overexpression of epidermal growth factor receptor and cyclooxygenase-2

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Abstract. A new human malignant rhabdoid tumor (MRT) cell line (designated FRTK-1) was established from MRT of the kidney of an 18-month-old boy. The cell line is maintained for over 24 months with more than 100 passages. FRTK-1 cells in vitro showed 2 different growth patterns, adherent and non-adherent patterns. The FRTK-1 cells showed the same morphological and immunophenotypical characteristics as primary tumor cells of kidney. Cytogenetic and molecular analyses revealed a non-sense mutation in the hSNF5/INI1 gene and loss of expression of hSNF5/INI1 gene product protein. Epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) were expressed in the FRTK-1 cells. Until now, there has been no report of MRT cell lines with expression of COX-2. Therefore, FRTK-1 cell line might be useful for investigating biological behavior and developing new molecular targeting antitumor drugs for MRT with expression of EGFR or COX-2.

Introduction

Malignant rhabdoid tumors (MRTs) are very rare and highly aggressive soft tissue sarcomas that occur in childhood, especially in infancy. In 1978, Beckwith and Palmer described renal MRT (MRT of the kidney; MRTK) as a rhabdomyosarcomatoid variant of Wilms' tumor (1). The tumor cells, designated as a ‘rhabdoid cell’, are histopathologically characterized by existence of an eosinophilic intracytoplasmic paranuclear inclusion consisting of whorls of intermediate filaments. MRT and its associated tumors occur most frequently in the kidney and also in other sites including the central nervous system (i.e., atypical teratoid/rhabdoid tumor; AT/RT), soft tissues, liver and chest wall. Abnormalities of the hSNF5/INI1 gene have been reported in MRT and its associated tumors, however, the rhabdoid cells show no evidence of actual rhabdomyoblastic differentiation and the origin still remains unclear.

MRTs frequently show highly aggressive behavior, resistance to multi-agent chemotherapy, and fatal metastasis (2). Therefore, in order to improve aggressive course or prognosis, molecular genetic and biological characterizations are required.

To our knowledge, 16 permanent MRT cell lines including 8 MRTK (3-8), 7 extrarenal MRT (6,9-12) and 1 AT/RT (13) have been previously established and characterized. However, these cell lines have not been investigated enough in the aspect of molecular genetic and biological behavior. In the present study, we have established and characterized a new cell line, FRTK-1, derived from MRTK and have investigated overexpression of epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) on FRTK-1 to clarify the biological behavior of MRTs.

Materials and methods

Case report. An 18-month-old Japanese boy presented with macrohématuria. Clinical examination revealed a left upper abdominal mass, and computed tomography showed a primary tumor arising from the left kidney and multiple lung metastases. The left renal tumor was totally resected. Light microscopically, the tumor of the left kidney showed a solid proliferation of small round cells with eosinophilic intracytoplasmic inclusions (Fig. 1). Mitotic figures were frequently seen. On electron microscope, the tumor cells had paranuclear aggregates or whorls of intermediate filaments, which were
light microscopically observed as intracytoplasmic inclusions (Fig. 2). Immunohistochemically, the tumor cells were positive for vimentin, neurofilament (NF), epithelial membrane antigen (EMA) and cytokeratins. As a result of pathological findings, the tumor was diagnosed as an MRTK. Two weeks after the operation, followed by adjuvant chemotherapy [etoposide, carboplatin, ifosfamide and pirarubicin]. However, local recurrence, liver metastasis and dissemination of peritoneum, as well as development of multiple lung metastases were found. The patient died of widespread tumor metastasis 3.5 months after the operation at the age of 23 months.

Establishment of cell line. The tumor cells were obtained from the surgical specimen of the left kidney. The cells were cultured at an initial concentration of 1.0x10^6 viable cells/ml in RPMI-1640 medium (Sigma R8758, St. Louis, MO, USA) supplemented with 15% heat-inactivated fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA), 50 units/ml penicillin G, and 50 μg/ml streptomycin. They were inoculated into 25-cm² tissue culture flasks (Iwaki Glass, Tokyo, Japan) and incubated at 37˚C in a humidified atmosphere with 5% CO2. The culture medium was changed twice per week. To harvest and transfer the cell line, the cells in the sub-confluent state were treated with 1000 unit/ml Dispase (Sanko-Junyaku, Tokyo, Japan). The cell line was maintained for over 2 years under these culture conditions.

Morphological study. The growth and in vitro morphology of cultured cells were observed with an inverted microscope. Morphologic characteristics were further determined by May-Giemsa staining on cytospin preparations and hematoxylin-eosin staining on paraffin-embedded sections. In addition, a transmission electron microscopic examination was done according to previously established procedures.

Immunohistochemistry. Immunohistochemical analysis was performed on the primary tumor, cultured cells and heterotransplanted tumors using the streptavidin-biotin complex (SABC) method. The antibodies were as follows: vimentin (clone V9, 1:1000 dilution; Dako, Glostrup, Denmark), NF (clone 2F11, 1:100 dilution; Dako), cytokeratins including AE1/AE3 (clone AE1 and AE3, 1:200 dilution; Dako), EMA (clone E29, 1:100 dilution; Dako), muscle-specific actin (MSA; clone HHF35, 1:1000 dilution; Dako), desmin (clone D33, 1:20 dilution; Dako), S-100 protein (polyclonal, 1:2000 dilution; Dako), neuron-specific enolase (NSE; polyclonal, 1:3 dilution; Nichirei, Tokyo, Japan), CD99 (MIC-2; clone 12E7, 1:200 dilution, Dako), Ki-67 (MIB-1; clone MIB-1, 1:300 dilution; Dako), p53 gene product protein (p53; clone DO-7, 1:150 dilution; Dako), hSNF5/INI1 (clone 25, 1:200 dilution; BD Biosciences, Franklin Lakes, NJ, USA), EGFR (clone EGFR.25, 1:100 dilution; Novocastra, Newcastle, UK) and COX-2 (clone 4H12, 1:100 dilution; Novocastra).

Cell population doubling time. To determine the doubling time, 2.0x10^5 cells were seeded on 3.5 cm plastic dishes (BD Falcon 353046, BD Biosciences) with fresh culture medium containing 15% FCS. The dishes were harvested, and the number of viable cells in each dish was counted by the dye exclusion test (0.1% trypan blue in phosphate-buffered saline; PBS) every 12 h for 5 days.

Heterotransplantation. Severe combined immunodeficiency (SCID) mice (lcr/scid female) and athymic nude mice (BALB/cA-nu/nu female) at age of 7 weeks were purchased from CLEA Japan (Tokyo, Japan) and kept under sterile conditions. Animal experiments were carried out under the control of our committee in accordance with the guidelines on Animal Experiments in Fukushima Medical University, Japanese Government Animal Protection and Management Law (No. 105) and Japanese Government Notification of Feeding and Safekeeping of Animals (No. 6). The mice were given a single subcutaneous or intraperitoneal injection of 2.5x10^7 cells from the cultured cells in the log phase of growth.

Cytogenetics. Karyotype analysis of the primary tumor cells and established cells was performed commercially by SRL (Tokyo, Japan) using trypsin G-banding technique.

DNA sampling. DNA sample was extracted from the cultured cells using Sepagene (Sanko-Junyaku) according to the manufacturer’s protocol.
Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and direct sequencing analysis for p53 gene. Genomic PCR-SSCP in exons 5-8 of the p53 gene, which are considered to be the mutational hot spot of p53 gene, was performed on FRHK-1 cells (SRL). Moreover, oligonucleotide sequences in exons 5-9 of the p53 gene were analyzed by direct sequencing. Using Takara Ex Taq HS polymerase (Takara Bio Inc., Shiga, Japan), 0.5 μg of DNA amplified in a total volume of 50 μl. The sequencing primers were as follows (14, 15); exon 5-6 (sense): 5'-TTCC TCTTCTGAGTACTC-3'; exon 5-6 (antisense): 5'-AGGT GCAAAACGACCTCAG-3'; exon 7 (sense): 5'-GTGTGGTC CTCTATTGTTGGC-3'; exon 7 (antisense): 5'-CAAGTGGC TCCTGACCTGGA-3'; exon 8-9 (sense): 5'-CCTATCTCTGA GTAGTGTTA-3'; exon 8-9 (antisense): 5'-CCAAAGACT TATGACCTGAAG-3'. These primers were obtained from Sigma-Aldrich Japan K.K.. PCR reaction products were electrophoresed on a 20% polyacrylamide gel and visualized using the ECL Western blotting detection reagents and analysis system (Amersham). The proteins were visualized using the ECL Western blotting detection reagents and analysis system (Amersham). HBL-3, which was the human acute lymphoblastic leukemia cell line established in our laboratory (17), was served as a positive control for hSNF5/INI1.

Direct sequencing analysis for hSNF5/INI1 gene. The mutation analysis was carried out in 9 exons of the hSNF5/INI1 gene. Direct sequencing of 9 exons was performed in FRHK-1 cell line. Using Takara Ex Taq HS polymerase, 0.5 μg of DNA were amplified in a total volume of 50 μl. The sequencing primers were the same as previously described (16), and obtained from Sigma-Aldrich Japan K.K.. PCR was performed as follows: denaturing at 94°C for 3 min followed by 30 cycles of amplification (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 10-min extension at 72°C, in a thermal cycler (i-cycler, Bio-Rad Laboratories Inc., CA, USA). One microliter of the PCR product was applied for PCR under the condition consisting of 1 cycle of 95°C for 5 min and 25 cycles of 95°C for 30 sec and 60°C for 30 sec by the direct sequencing method (Thermo Sequenase core sequencing kit with 7-deaza-dGTP; Amersham, OH, USA). Subsequently, oligonucleotide sequences of exons 5-9 of the p53 gene were analyzed using a sequencer (SQ-5500, Hitachi, Tokyo, Japan) and compared with the germline sequences recorded in the GenBank database.

Western blot analysis for hSNF5/INI1 gene product protein, EGFR and COX-2. Cells grown in 25-cm² tissue culture flask were washed twice with ice-cold 0.01 M PBS and scraped in ice-cold 0.01 M PBS, and the cell pellets were lysed in 1 ml of lysis buffer (20 mM Tris-HCl pH 7.4, 1% NP-40, 1 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 0.05 mM Na3VO4, 0.1 mM PMSF, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin) at 4°C for 1 h and clarified by centrifugation (1200 rpm for 15 min). Twelve microliter of the supernatant was suspended in 3 μl of sample buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% 2-mercaptoethanol and 2.3% SDS. The samples were subjected to SDS-PAGE (13% of polyacrylamide gel for hSNF5/INI1 gene product protein and COX-2, and 7.5% of polyacrylamide gel for EGFR) under reducing conditions at a constant current of 20 mA. The separated proteins were transferred to PVDF membrane (Millipore, Tokyo, Japan) at a constant current of 300 mA for 60 min. To block non-specific binding, the membrane was incubated with blocking buffer (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The membrane was washed with washing buffer, and incubated with the antibodies of hSNF5/INI1 (clone 25, 1:250 dilution; BD), EGFR (1005; sc-03, 1:1000 dilution; Santa Cruz, CA, USA) and COX-2 (H-62; sc-7951, 1:200 dilution; Santa Cruz). After washing, the membrane was incubated with 1 μg/ml anti-mouse or anti-rabbit immunoglobulin (Bio-Rad Laboratories Inc.). The proteins were visualized using the ECL Western blotting detection reagents and analysis system (Amersham). HBL-3, which was the human acute lymphoblastic leukemia cell line established in our laboratory (17), was served as a positive control for hSNF5/INI1.

Results

Immunohistochemical analysis for p53, hSNF5/INI1, EGFR and COX-2 on primary tumor cells. Immunohistochemically, the tumor cells were positive for p53, EGFR and COX-2, but not for hSNF5/INI1 (Table I). In contrast, 2 cases of Wilms' tumor, 2 cases of clear cell sarcoma of the kidney, 2
cases of congenital mesoblastic nephroma and 2 cases of malignant lymphoma were all positive for hSNF5/INI1 (data not shown).

Establishment of cell line. A new human malignant rhabdoid tumor cell line, designated FRTK-1, was established from the left kidney of a patient with malignant rhabdoid tumor. The doubling time of the cell line was approximately 24 h. The cell line has been stably maintained for over 100 passages. More than half of the cultured cells in vitro were adherent and the others were non-adherent. The adherent cells were spindle-shaped, stellar or multilateral-shaped, and loss of contact inhibition was observed. On the other hand, the non-adherent cells showed the round shape (Fig. 3). Both adherent and non-adherent cultured cells showed rhabdoid features with eosinophilic intracytoplasmic inclusions and no difference in immunohistochemical results between them was found. Electron microscopically, the cultured cells also showed diffusely positive reaction for vimentin, neurofilaments, EMA, S-100 protein, Ki-67, p53 gene product and COX-2 (Fig. 5), and focally positive reaction for AE1/AE3, NSE, CD99 and EGFR, but were negative for MSA, desmin and hSNF5/INI1 (Table I).

Heterotransplantation. FRTK-1 cell line showed successful heterotransplantation into SCID mice and athymic nude mice. In 2 out of 2 SCID mice and 2 of 2 nude mice given a single intraperitoneal injection of FRTK-1 cells, many small nodular tumors developed in the surface of the liver, spleen, mesenterium and peritoneum 14 days after inoculation. The cut surface of the tumors was solid and white. Light microscopically, the tumors showed a solid proliferation of small round cells. There was no metastasis to the brain, lung or kidney and no direct invasions of the liver or spleen in one SCID mouse and 2 nude mice. However, in another SCID mouse, the tumor cells directly invaded the fibrous capsule of liver. Immunohistochemical study showed the same immunoprofile of the primary tumor cells and as the FRTK-1 cells (Table I).

Cytogenetics. Karyotype analysis of G-banded chromosomes for the primary tumor cells showed a reciprocal translocation between chromosomes 10 and 12 [46, XY, t(10;12) (q22; p13)] in 1 out of 21 cells. In FRTK-1 cell line, 3 of 12 cultured cells showed [46, XY, dup(2) (q23;q33)], and 1 cell each showed [47, XY, +mar] and [47, XY, add(7) (q32), +mar] (data not shown).

PCR-SSCP analysis for p53 gene. In FRTK-1 cell line, no genetic abnormalities were detected in exons 5-8 of p53 gene by PCR-SSCP, and no mutations of oligonucleotide sequences were detected in exons 5-9 of p53 gene by direct sequencing analysis (data not shown).
Direct sequencing analysis for hSNF5/INI1 gene. In FRTK-1 cell line, the direct sequencing method detected a point mutation of hSNF5/INI1 gene from GAG to TAG at codon 300, which resulted in conversion from glutamine to stop codon (non-sense mutation) in exon 7 (Fig. 6). No other abnormalities were detected.

Western blotting for hSNF5/INI1 gene product protein, EGFR and COX-2. FRTK-1 cells showed no expression of hSNF5/INI1 gene product protein but expressed EGFR and COX-2 protein (Fig. 7). HBL-3 cells as a positive control expressed hSNF5/INI1 gene product protein.

RT-PCR analysis for EGFR and COX-2 mRNA. Both EGFR and COX-2 mRNA were detected in FRTK-1 by RT-PCR analysis (Fig. 8).

Discussion

We established a new cell line (FRTK-1) derived from human MRTK. Morphological and immunophenotypic findings...
promoting the tumorigenesis of MRTK. Molecular genetic analysis revealed that FRTK-1 cell line had a non-sense mutation in exon 7 in hSNF5/INI1 gene. The aberrations of the hSNF5/INI1 gene (non-sense mutation at codon 300) in FRTK-1 cell line have not been previously reported. The hSNF5/INI1 gene is considered to be a tumor suppressor gene for MRTs (16,21-25) and reported to play a role of suppressing tumor growth in mouse models (26-29). Therefore, the non-sense mutation in exon 7 of FRTK-1 cell line may lead to inactivation of the hSNF5/INI1 gene, thus promoting the tumorigenesis of MRTK.

In 2004, Judkins et al (30) and Hoot et al (31) reported that disappearance of the hSNF5/INI1 protein was specific for AT/RT and MRTs regardless of abnormality in the hSNF5/INI1 gene. Therefore, detection of the hSNF5/INI1 protein by immunohistochemical staining is useful for diagnosis of MRTs including AT/RT, FRTK-1 cells both in vitro and in vivo expressed no hSNF5/INI1 protein, and also primary tumor cells were negative for hSNF5/INI1. Further more, hSNF5/INI1 protein was not detected in FRTK-1 by Western blotting. The data suggest that the non-sense mutation of the hSNF5/INI1 gene leads to disappearance of the hSNF5/INI1 protein and fails to suppress tumorigenesis of MRT.

Kinoshita et al (32) have reported that 5 out of 6 cases of MRTs (3 of 3 renal, and 2 of 3 extrarenal MRTs) showed mutation of the p53 gene, which is one of the most common tumor suppressor genes in various human neoplasms, and suggesting the relevance to its highly aggressive behavior. On the other hand, Rosson et al (6) noted no gene mutation in the p53 gene in 5 MRT cell lines (2 renal and 3 extrarenal MRTs). In FRTK-1, no genetic abnormalities were detected in exons 5-9 of the p53 gene. Thus, in FRTK-1, the hSNF5/INI1 gene might mainly behave as a tumor suppressor gene and the p53 gene might have little or no relation with tumorigenesis.

EGFR is a receptor tyrosine kinase and is widely expressed in a variety of epithelial malignancies (33). EGFR activation promotes tumor growth by increasing cell proliferation, motility or angiogenesis, and by blocking apoptosis (34). In MRTs, EGFR expression and cell growth inhibition by anti-EGFR antibody in vitro (17) or by selective EGFR tyrosine kinase inhibitor, Gefitinib (ZD1839, Iressa), in vitro and in vivo (35) have been reported. Therefore, in view of the molecular targeting therapy, EGFR is recognized as a new target for the therapy of MRTs.

COX, also known as prostaglandin H2 synthase or prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostanooids (36). Two COX genes are cloned; COX-1 is a constitutive enzyme produced constantly in most tissue types, and is probably responsible for the production of prostanooids under physiological conditions, COX-2 is undetectable in most normal tissues, but can be induced in various cell types by pro-inflammatory agents, growth factors and carcinogens (37). Overexpression of COX-2 has been found in various malignancies including carcinomas, lymphomas and some sarcomas. In addition, COX-2 activation promotes tumor growth through production of prostaglandin E2 that could block apoptosis or activate vascular endothelial growth factor and angiogenesis (38). Recent studies have showed antitumor effect of some selective COX-2 inhibitors for sarcomas (39,40) as well as carcinomas. However, COX-2 expression in MRTs has not been reported.

FRTK-1 cells expressed both EGFR and COX-2. Crosstalk between EGFR and COX-2 has been found, and cooperative cell-growth inhibition by the combination treatment with a selective EGFR tyrosine kinase inhibitor and a selective COX-2 inhibitor in malignancies is expected to be efficacious (41). Therefore, FRTK-1 cell line is useful for investigating the antitumor effect of EGFR tyrosine kinase inhibitor combined with COX-2 inhibitors.

In conclusion, we established a new cell line (FRTK-1) derived from MRTK. The FRTK-1 cells showed the same morphological and immunophenotype as those of the primary tumor cells which are characteristic of MRTK, a non-sense mutation of the hSNF5/INI1 gene in exon 7, and expression of EGFR and COX-2 protein. FRTK-1 cell line is useful for investigating biological behavior and developing antitumor therapy of MRT.

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References


