Abstract. Hepatoid adenocarcinoma is a rare gastrointestinal tumor and mostly reported in the stomach. Effective chemotherapy has yet to be developed to improve poor prognosis. The present study was undertaken to establish a useful cell line derived from a hepatoid adenocarcinoma, possibly leading to a new therapeutic strategy. The new human cell line VAT-39 was established from a metastatic lymph node of a 69-year-old Japanese male patient with hepatoid adenocarcinoma of the ampulla of Vater. The primary tumor and metastatic lymph node were composed of hepatoid adenocarcinoma cells exhibiting immunohistochemical reactivity for alpha-fetoprotein (AFP) and glypican-3 (GPC3). In the metastatic lymph node, Periodic acid-Schiff (PAS) staining clarified diffuse deposition of glycogen in the cytoplasm, indicating analogous characteristics to the primary hepatoid adenocarcinoma. Moreover, VAT-39 cells produced high levels of AFP in the cultured medium, and reverse-transcriptase polymerase chain reaction (RT-PCR) verified increased expression of GPC3 mRNA in this cell line. Further, we evaluated the sensitivity to major chemotherapeutic drugs against the bile duct cancer. Neither 5-fluorouracil nor gemcitabine showed particular sensitivity to this cell line. The tumorigenicity of the cultured cells was confirmed in athymic nude mice and the histological features of the explanted tumor were similar to the VAT-39 cell line. The present VAT-39 is the first hepatoid adenocarcinoma cell line that originates from the ampulla of Vater and it will be applicable for basic biological studies searching for new strategies of molecular targeted chemotherapy to this disease.

Introduction

Hepatoid adenocarcinoma is a rare gastrointestinal tumor and mostly reported in the stomach. The first case of α-fetoprotein (AFP)-producing gastric carcinoma was reported in 1970 by Bourreille et al (1). It has been generally accepted to categorize the hepatoid adenocarcinoma as gastric carcinoma with a feature of hepatic differentiation complicated with high serum AFP level, proposed by Ishikura et al (2). Besides the stomach, to our knowledge, it has also been described in colon (3), pancreas (4), lung (5), esophagus (6), papilla of Vater (7), urinary bladder (8), renal pelvis (9), ovaries (10), uterus (11) and the cervix (12).

Hepatoid adenocarcinoma is pathologically diagnosed by morphological similarity to the hepatocellular carcinoma (HCC), and immunohistochemical reactivity for AFP and remarkable elevation of serum AFP are crucial characteristics to the diagnosis. Clinically, hepatoid adenocarcinoma shows particular characteristics as metastasis to the liver and lymph nodes. Additionally, effective chemotherapy has not been established as yet suggesting poor prognosis. More efficient strategy for the treatment of this malignant tumor is necessary since it is not so rare. The incidence rates of 2.7-5.4% (13-15), of AFP-producing primary gastric carcinoma indicates the infrequency of hepatoid adenocarcinoma. It should be noted that the currently used AFP-producing hepatoid adenocarcinoma cell lines, Takigawa (16), FU 97 (17), ISt-1 (18), OSS and TTS (19), were derived from the stomach. In considering various sites of primary tumor and its malignant characteristics, new cell lines derived from non-gastric cancers are required to develop new strategies.

Herein we describe the establishment of a novel hepatoid adenocarcinoma cell line, designated as VAT-39, and its biological characteristics. This cell line will be a useful tool to clarify further characteristics of hepatoid adenocarcinoma and to develop a new treatment strategy for this refractory disease.
Materials and methods

Primary culture. A 69-year-old Japanese man consulted a local doctor because of a right flank pain. He was referred to our hospital in 2009 for further examination and treatment. Laboratory values upon admission indicated liver dysfunction and obstructed jaundice. Endoscopic retrograde cholangiopancreatography and abdominal computed tomography suggested adenocarcinoma of the ampulla of Vater with lymph node metastasis. No metastasis to the liver and the other organs was observed. On the basis of these findings, pylorus-preserving pancreatoduodenectomy with lymph node dissection was performed (Fig. 1A). Pathological diagnosis was hepatoid adenocarcinoma of the ampulla of Vater with lymph node metastasis (Fig. 1B) considering the positive immunoreactivity for AFP (Fig. 1C). Small part of the viable lesion of the lymph node metastasis was resected surgically during an operation, rinsed with phosphate-buffered saline (PBS), and minced with scissors, followed by digestion in 1 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1000 PU/ml dispase (Godo Shusei, Tokyo, Japan) in the mixture of RPMI-1640 (Nissui, Tokyo, Japan) and Ham's F12 (Nissui) at the rate of 1:1 at 37°C for 4 h. The remaining part was used for pathological diagnosis. Then the solution was centrifuged at 700 rpm for 3 min and the pellet was resuspended in the same medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, KS, USA), 100 IU/ml penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 100 µg/ml streptomycin (Meiji Seika Pharma Co., Ltd.), 20 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) (Nakalai, Kyoto, Japan), pH 7.4. The flasks were cultured in a fully humidified atmosphere of 5% CO₂ in air at 37°C. Informed consent was obtained from this patient and the protocol was approved by the ethics board of the Faculty of Medicine, University of Miyazaki. Mycoplasma infection was examined by a PCR Mycoplasma Detection kit (Takara Bio Inc., Shiga, Japan).

Growth characteristics. Growth curves were established by seeding 1x10⁵ cells/5 ml in growth medium onto 60-mm culture dishes. Triplicate dishes were harvested and counted daily. Throughout the entire procedure, cell viability was determined by means of the trypan blue exclusion method. Doubling time was determined during the exponential phase of growth.

Karyotyping and short tandem repeat (STR) analysis. Exponentially growing cultures of the cell line, passage number 50, was harvested for chromosome preparations with standard procedures by SRL (Tokyo, Japan). Fifty cells were analyzed for chromosome number and the Giemsa banding technique for karyotype analysis. STR profiling at the passage number 1 and 50 were performed by Takara Bio Inc. to identify the cross-contamination of another cell line.

Morphological analysis. For light microscopy, VAT-39 cells were cultivated on Chamber Slide (Nunc Inc., IL, USA) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min at room temperature (RT). After washing with PB, the cells were observed with hematoxylin-eosin (H&E) or Periodic acid-Schiff (PAS) staining to examine their morphology and the existence of glycogen, respectively.

For electron microscopy, high-pressure freezing (HPF) technique was applied for fine structural observation (20,21). In brief, VAT-39 cells were cultivated on sterilized 10 µm-thin stainless foil (YUS205-M1, Nippon Steel, Tokyo, Japan). At semi-confluency, the attached VAT-39 cells on the stainless foil were immediately cryofixed in the HPF machine (HPM010, BAL-TEC, Liechtenstein). The assembly with cryofixed cells was transferred to 1 ml of freeze substitution medium (1% osmium tetroxide in acetone), and then freeze substituted in a Leica AFS machine (Leica, Vienna, Austria). After embedding into epoxy resin, ultrathin sections (60-80 nm) were cut and stained with uranyl acetate and lead.
citrate to be observed in a transmission electron microscope (HT7700, Hitachi, Tokyo, Japan).

Electron microscopic localization of cytoplasmic glycogen was performed by means of periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method. Briefly, the VAT39 cells cultivated on the micro-cover glass (thickness no. 5, Matsunami, Osaka, Japan) (22) were fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB for 2 h at RT. After washing with PBS, without post-fixation by 1% osmium tetroxide, the cells were dehydrated and embedded in epoxy resin. Ultrathin sections mounted on gold meshes were treated in 1% sodium periodate (Wako Pure Chemical Industries, Ltd.) for 30 min followed by 1% thio-carbohydrazide (Sigma-Aldrich, MO, USA) for 1 h. After washing, the sections were incubated in 1% silver protein (Merck, Germany) for 1 h and observed as described above.

**Immunohistochemistry of AFP.** Conventionally prepared paraffin sections were deparaffinized, and incubated in 0.3% H$_2$O$_2$ in methanol to block endogenous peroxidase for 30 min. After rinsing in PBS, the sections were treated with 1% bovine serum albumin (BSA) in PBS for 20 min, and then incubated with a polyclonal rabbit anti-human AFP (Dako, Tokyo, Japan; 1:10 diluted with 1% BSA in PBS) for 1 h at RT. After washing with PBS, the sections were incubated with biotinylated horse anti-rabbit IgG (Vector Laboratories, CA, USA; diluted 1:300 with 1% BSA in PBS) for 40 min at RT. After washing with PBS, the sections were incubated in a freshly prepared solution of avidin-biotinylated horse-radish peroxidase complex (ABC) kit (Vector Laboratories) for 30 min (23), and then peroxidase reaction was developed by incubating in 0.05% 3,3-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, pH 7.6, containing 0.001% H$_2$O$_2$ for 5-10 min. After washing, the sections were briefly counterstained with hematoxylin.

**Detection of AFP in the serum and the culture medium.** The sera, preoperative and postoperative conditions, were collected as a routine follow up of operation. The supernatants of VAT-39 at passage 50 were harvested after 24, 48 and 72-h culture in 5 ml of serum-free media in a 25 cm$^2$ flask. The AFP concentrations in these samples were measured by Architect AFP using Archicteco i200SR (Abbott, Chiba, Japan).

**RT-PCR of HCC related gene.** Human biliary tract cancer cell lines (HuCCt1, HuH-28, TFK-1, TKKK, TGB2C2TKB, TGBC14TKB and TGBC18TKB) were obtained from Riken Cell Bank (Tsukuba, Japan). The two other human biliary tract cancer cell lines (IHGGK and G415) were obtained from Tohoku University (Miyagi, Japan). Total RNAs were extracted with TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), followed by Cloned DNase I (Takara Bio, Inc.) treatment and phenol/chloroform/isoamylalcohol extraction. For RT-PCR, 6 µg of total RNA was reverse transcribed with a mixture of oligo (dT) and random primer using 200 units of ReverTra Ace® (Toyobo, Osaka, Japan), and 1/90 of the resultant cDNA was processed for each PCR with 0.25 µM of both reverse and forward primers and 1.25 units of HotStar™ Taq DNA polymerase (Qiagen, Tokyo, Japan). For detection of hepatoid adenocarcinoma-related gene mRNA, the following primers were designed: AFP, sense 5'-GACATCCTCAGGTTGCT GTC-3' and antisense 5'-GAGGCCGAGCTTCGCTTTTG-3' (173 bp); GPc3, sense 5'-GCAGGAAAAGTGGCACACCAC-3' and antisense 5'-GACATGTCCTCAGGAGCTTG-3' (419 bp); glyceralddehydes-3-phosphate dehydrogenase (GAPDH), sense 5'-GTGAAGTGCGAGTCAAG-3', and antisense 5'-GTT GAAGCGCCAATGGACTC-3' (300 bp). The PCR products were analyzed by 1.5% agarose gel electrophoresis.

In vitro sensitivity to chemotherapeutic agents. We evaluated the effect of several chemotherapeutic reagents, generally used to the patient of biliary tract cancer, on the growth of VAT-39 at the passage number 50. Gemcitabine, 5-fluorouracil, epirubicin and mitomycin C were purchased from Wako Pure Chemical Industries, Ltd.; paclitaxel, oxaliplatin and doxorubicin were from Nakalai; cisplatin was from Sigma-Aldrich. The VAT-39 cells (1x10$^5$) were seeded in 96-well plates and cultivated overnight in 100 µl of the same medium containing 10% FBS. The medium was removed and replaced with the same medium containing various concentrations of the chemical agents. After 24 h incubation, 10 µl of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was added to each well. Following further 3 h incubation, the absorbance at 450 nm was monitored using ImmunoMini NJ-2300 (Biotec Co. Ltd., Tokyo, Japan).

In vivo experiment. Tumorigenicity and metastatic potential of VAT-39 cells were analyzed using 6 weeks old BALB/c athymic mice (Kyudo, Kumamoto, Japan). After trypsinization of cultured cells by 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (TE, Sigma-Aldrich), the cells were centrifuged immediately at 1000 x g for 5 min, and the resuspended cells (1x10$^5$ in 0.2 ml PBS) were injected subcutaneously at the abdominal flank. At 8 weeks or when the mice became moribund, they were sacrificed or necropsied. Under general anesthesia using diethyl ether, the xenograft, brain, lung, liver, kidneys and gastrointestinal tract were harvested and examined under a dissecting microscope. Tumor volume was calculated weekly using the formula, length x (width)$^2$ x 0.5. For light microscopy, xenografted tumors were fixed with 4% paraformaldehyde in 0.1 M PB at 4°C overnight, and embedded in paraffin. The sections were stained with H&E.

All animal procedures were carried out under protocols approved by the University of Miyazaki Animal Research Committee, in accordance with international guiding principles for biomedical research involving animals.

**Results**

Establishment of the VAT-39 cell line and light microscopic characterization. The tumor cells formed colonies surrounded by fibroblasts in two months of primary culture. The fibroblasts were eliminated by a weak trypsin and TE treatment for one month. The first passage was done at a split ratio of 1:4. The cell line was designated as VAT-39, and it has already undergone more than 100 passages. The VAT-39 cells proliferated well in culture media containing 10% FBS. The polygonal cells with prominent nucleoli formed adhesive sheet-structures as shown in phase-contrast microscopy (Fig. 2A). Morphological findings of
VAT-39 (Fig. 2B) were mostly in accordance with those of
the lymph node metastasis (Fig. 1B) in which mitotic figures
were frequently seen. Growth characteristics were determined
at passages 1 and 50. The doubling times at passage 50 were
34 h (data not shown). The cell line was free from mycoplasma
infection.

**STR and karyotype analysis.** The STR analysis was performed
at passages 1 and 55 of the VAT-39 cells, and the obtained
STR profile ~10 loci and detected peaks were: AMEL (X),
CSF1PO (12), D13S317 (11), D16S539 (9,12), D21S11 (30),
D5S818 (12), D7S820 (12,13), TH01 (9), TPOX (8,11), vWA
(14,17). The STR analysis at passage 1 was identical to that
of 55, which confirmed that VAT-39 cell lines maintained the
same genetic feature without possible contamination.

The number of chromosomes was between 50-54 at passage
50 with a modal chromosome number 53. The result of karyo-
type analysis of 10 cells were as follows: X, -Y, +add(1)(p11),
add(1)(q21), +del(3)(p11), der(3)add(3)(q21)del(3)(q?), add(7)
(p11.2), add(9)(p13), der(11)add(11)(p11.2)add(11)(q23), der(11)
add(11)(p15)add(11)(q23), add(13)(p11.2)x2, -14, +del(16)(p?),
del(16)(q?), add(19)(p13), -20, add(21)(q22), +mar1, +mar2,
+mar3, +mar4x2. No change was observed in chromosome
number 4 and X where the AFP and GPC3 genes are located,
respectively.

Transmission electron microscopy and detection of glycogen
granules. Transmission electron microscopic study by HPF
revealed electron lucent granules in the cytoplasm of VAT-39
cells (Fig. 3A), suggesting accumulations of glycogen gran-
ules. Light microscopy by PAS staining demonstrated strongly
stained numerous granules in the cytoplasm (Fig. 3B). At
the electron microscopic level, PA-TCH-SP method clarified
the accumulation of glycogen in the electron lucent granules
(Fig. 3C).

**AFP expression in the cells, culture media and sera.** Immunohistochemistry of AFP demonstrated a distinct
immunoreactivity around the perinuclear region of VAT-39
cells (Fig. 4A). The expression of AFP mRNA in VAT-39
cells was evaluated by RT-PCR (32 cycles of amplification), in
comparison with various cell lines derived from biliary tract
cancer. As a result, the VAT-39 cell line exceptionally expressed
AFP mRNA (Fig. 4B). The expression was confirmed in the
lymph node metastasis, from which the VAT-39 cell line was
established, as well as in the VAT-39 cells of varying passage
numbers (Fig. 4C). Moreover, the VAT-39 cell line and the
lymph node metastasis also expressed GPC3 mRNA (Fig. 4B
and C) that is highly expressed in HCC. The AFP protein
concentrations were highly elevated in the culture media
depending on the culture period (Table IB).

Although the AFP protein concentration in the preoperative
serum indicated a remarkably high level, the concentration was
drastically decreased in the postoperative serum (Table IA).
Taken together, the elevated serum AFP was considered to be secreted from the tumor, and this phenotype was well-preserved in this established cell line.

Chemotherapeutic sensitivity. The VAT-39 cells were subjected to various chemotherapeutic reagents to evaluate their sensitivities to each reagent concerned with 50% inhibition concentration (Table II). As a result, the VAT-39 cell line exhibited no sensitivity to 5-FU and gemcitabine.

Transplantation to the nude mouse. Tumorigenicity was tested by subcutaneous injection of VAT-39 cells at the abdominal flank of nude mice. The cells grew rapidly to form solid tumor masses, which were detected within 4 weeks (Fig. 5A, inset). The tumor doubling time was calculated as 2.5 days (data not shown). The VAT-39 cell pellets showed 100% tumorigenicity in all the transplanted mice (n=5; data not shown). It should be noted that obvious tumor mass necrosis was seen on a cut surface of the xenografted tumor. Histopathology demonstrated central necrosis, surrounded by viable polygonal tumor cells in the peripheral region. The viable cells formed sheet-structures, showing hyperchromatic nuclei, prominent nucleoli, foamy and clear cytoplasm, and numerous mitotic figures (Fig. 5A). The expression of AFP was also noted in the explanted tumor of the nude mouse (Fig. 5B), preserving the characteristics of AFP expression. These findings were consistent with the original tumor and lymph node metastasis (Fig. 1B). In the explanted mice, no metastatic lesion was identified in the other organs.

Discussion

Hepatoid adenocarcinoma is a rare gastrointestinal tumor and mostly reported in the stomach. Ishikura et al (2) proposed to

Table I. Concentrations of AFP in preoperative, postoperative sera and culture media.

<table>
<thead>
<tr>
<th>A, Sera</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Concentration (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>Preoperation</td>
<td>1,955</td>
</tr>
<tr>
<td>Postoperation (12 days)</td>
<td>255</td>
</tr>
<tr>
<td>Postoperation (28 days)</td>
<td>46.4</td>
</tr>
</tbody>
</table>

| B, Culture media |
|------------------|----------|
| Media            | Concentration (ng/ml) |
| Serum-free medium | 0.4      |
| Non-cultured medium + 10% FBS | 1.8      |
| Cultured medium (after 24 h of incubation) | 7,370    |
| Cultured medium (after 48 h of incubation) | 10,106   |
| Cultured medium (after 72 h of incubation) | 12,722   |

Figure 4. Detection of AFP and GPC3. (A) Immunohistochemical localization of AFP in VAT-39 cell line cultured on the chamber slide. (B and C) RT-PCR of AFP and GPC3 mRNA. (B) Note the expression of AFP and GPC3 mRNA by VAT-39 cell lines among the 10 biliary tract cancer cell lines. (C) AFP and GPC3 expressions are shown in the primary lymph node metastasis and VAT-39 cell line at passages 1 and 50.

Figure 5. In vivo experiments. (A) H&E stain of the xenograft cells explanted in the abdominal flank of athymic mice (inset). (B) Immunohistochemical localization of AFP in the explanted tumor.
elucidated an angiogenesis-dependent behavior of AFP-producing gastric carcinoma. In fact, treatment with anti-AFP antibody provided down-regulation of angiogenesis, suggesting that AFP itself is likely to upregulate angiogenesis, and the anti-angiogenic effects by antibody treatment may inhibit metastasis, especially to the liver (19). It has been reported that AFP-positive group showed higher incidence of lymph node metastasis, deeper invasion into the gastric wall, higher frequency of advanced stage, marked lymphatic invasion, and higher rate of liver metastasis in comparison to AFP-negative group (13). Another study reported that liver metastasis occurred in 72% of AFP-positive patients whose mortality reached 100% within 2 years (15). Although the function of AFP is not completely understood, a control of AFP expression may improve the poor prognosis. The VAT-39 cell line retains a large amount of AFP production, similar to Takigawa and FU97 cell lines (16,17), even after 50 passages. Therefore, the VAT-39 cell line would be a useful tool for elucidating the function and expression of AFP to develop a new therapeutic strategy.

It has been recently reported that circumferential membranous GPC3 immunoreactivity in HCC indicated a poor prognosis particularly in patients with hepatitis C virus infection (27). The GPC3 is thought to be a sensitive marker for hepatoid adenocarcinoma (28), and the level of GPC3 induction is supposed to be controlled by alpha-fetoprotein regulator 2 (Afr2) (29). A monoclonal antibody, named GC33, was recently developed against human GPC3 (30). The GC33 antibody recognizes a GPC3 ectodomain and provides significant antitumor activity in vivo by inducing antibody-dependent cellular cytotoxicity (30,31). Although expression of neither AFP nor GPC3 mRNA was identified in the bilia tract cancer cell lines, the present VAT-39 cell line exhibited extraordinary expression of both AFP and GPC3 mRNA as high as the original lymph node metastasis used for the primary culture. In this connection, the GC33 might be a candidate for promising antitumor antibody to improve the poor prognosis of hepatoid adenocarcinoma, and the VAT-39 cell line would be a useful tool to examine any potential effect of GC33 on this refractory disease.

In summary, the present establishment and biological characterization of VAT-39 cell line will provide a novel experimental model for biological investigations searching for a new therapy to treat hepatoid adenocarcinoma.

Acknowledgements

We thank Yoshiteru Goto (Division of Electron Microscopy in Frontier Science Research Center, University of Miyazaki) and Yasuyo Todaka (Department of Anatomy, Ultrastructural Cell Biology, Faculty of Medicine, University of Miyazaki) for their expert assistance. This study was supported by Grants-in-Aids for Scientific Research from the Japan Society for the Promotion of Science (nos. 23791552 and 30336292).

References


