Establishment and Characterization of Human Signet Ring Cell Gastric Carcinoma Cell Lines with Amplification of the c-myc Oncogene

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ABSTRACT

Two unique human signet ring cell gastric carcinoma cell lines (designated HSC-39 and HSC-40A) were established in vitro from the ascites of a 54-year-old male patient. Both cell lines were biologically quite similar, grew in vitro in suspension with a population doubling time of 28–30 h, and had cytohistological features of mucinous epithelial tumor cells. They formed colonies in soft agar, with a cloning efficiency of 0.8–1.0%. Ultrastructurally, numerous granules were observed in the cytoplasm, suggesting secretory activity. The frequent presence of desmosome and the tight junction at the cell boundary certifies the epithelial origin of the lines. Immunocytochemistry and radioimmunoassay showed production of tumor marker antigens (carcinoembryonic antigen, CA 19-9, and sialyl-Le^a^-i) and gastrin in both lines. These lines were transplantable in athymic BALB/c nude mice. The histopathology of each line growing in athymic BALB/c nude mice was similar to that of the original tumor. The karyotype of the cells was highly aberrant with structural and numerical changes. The presence of numerous double minute chromosomes and loss of the 13 chromosome and Y-chromosome characterize these lines. In addition, the amplified c-myc oncogene (16–32-fold) was found in both cell lines and original ascitic tumor cells. Overexpression of the c-myc mRNA was noted. These cell lines may be a useful tool, providing both in vitro and in vivo systems for further studies of the biology and therapy of human signet ring cell (or Borrmann’s type IV carcinoma) gastric carcinoma.

INTRODUCTION

Although the prevalence of human gastric carcinoma is gradually decreasing in recent years, it still is a significant cause of cancer-related deaths in Japan (1). Human scirrhous gastric carcinoma (diffusely infiltrating carcinoma or Borrmann’s type IV carcinoma) of the stomach possesses a number of unique biological properties such as extensive fibrosis with sparse tumor cell infiltration in demoplastic stroma and the worst clinical prognosis of any other types of gastric cancer (2, 3). The establishment of an in vitro model system is expected to be one of the desirable approaches to elucidate features of scirrhous gastric carcinomas. However, only one scirrhous gastric carcinoma cell line has been established from the pleural effusion of a 55-year-old Japanese male with a signet ring cell carcinoma (4).

We now report a successful establishment and characterization of gastric carcinoma cell lines derived from the ascites of a patient with signet ring cell gastric carcinoma. These lines have been analyzed with respect to the growth property, cellular ultrastructure, neoplastic behavior in athymic BALB/c nude mice, karyotype, and the expression of tumor-associated antigen. In addition, we found the amplification and overexpression of the c-myc gene in both cell lines. These lines should be useful for investigating the properties of signet ring cell gastric carcinoma cells and for the development and application of new therapeutic means as well.

MATERIALS AND METHODS

Origin of the Cells and Establishment of the Line. The cell lines were derived from malignant ascites taken by puncture from a 54-year-old Japanese male who had a gastric carcinoma. Clinical and histopathological diagnosis for the primary tumor was signet ring cell carcinoma (Borrmann’s type IV carcinoma). Chemotherapy was initiated because gastrectomy was not applicable. However, the tumor progressed, and the patient died of disseminated disease on June 1, 1989.

Ascites were collected under sterile conditions in a 500-ml bottle with heparin. The cells were harvested by centrifugation (1000 rpm for 10 min) and plated into 75-cm² culture flasks or 10-cm dishes containing Dulbecco’s modified Eagle’s medium/α-MEM (1:1) supplemented with 10% fetal calf serum (FCS), 5% horse serum, 2 mM l-glutamine, 100 μg/ml streptomycin sulfate, and 100 IU/ml penicillin G sodium. Media and sera were obtained from Grand Island Biological Co. (Grand Island, NY). No attempt was made to separate the malignant cell clumps from lymphocytes, macrophages, and erythrocytes.

The cells were incubated at 37°C in 5% CO₂ in air. The culture was observed regularly for the cell growth with a Nikon phase-contrast microscope. Subcloning was performed by limiting dilution in 96-well tissue culture plates. Resulting clones were passaged routinely at the split ratio of 1:10 or 1:20. Growth medium was composed of α-MEM supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml of streptomycin sulfate, and 100 IU/ml of penicillin G sodium. The two cell lines were routinely tested for Mycoplasma contamination and found to be negative, by staining with 4,6-diamino-2-phenolindole dihydrochloride fluorescence (Boehringer Mannheim, West Germany).

Growth Properties of the Cells. The doubling time of each line was determined as described previously (5). Briefly, 1 x 10⁵ cells were inoculated into 60-mm culture dishes and cell numbers were determined every day.

 Colony formation in semisolid agar was assayed as described previously (6) by plating 10⁴-10⁵ cells in α-MEM containing 10% FCS and 0.33 Difco Noble agar. The number of colonies formed were counted at 14 days after cell plating.

 Morphological, Histochemical, and Immunological Analysis. Cells were collected by centrifugation. The pellets were fixed in 10% formaldehyde, embedded in paraffin, and processed for histological examinations. Sections were stained with hematoxylin and eosin, PAS, or Alcian blue.

 HSC-39, HSC-40A, and normal stomach fibroblastic cells (2 x 10⁴) were seeded and cultured for 3 days. The medium was then replaced and the cell-free supernatant was collected 72 h thereafter to determine secreted products. Shedding of carcinoembryonic antigen, CA19-9, CA125, and sialyl-Le^a^-i antigen was tested by radioimmunoassay and immunoradiometric assay at Otsuka Assay Laboratories (Tokushima, Japan).

 Electron Microscopic Study. The cells were centrifuged, washed in cold phosphate-buffered saline (–), and fixed for 60 min with cold 1.25% glutaraldehyde. The cell pellets were thoroughly washed with cold phosphate-buffered saline (–) and postfixed in 2% OsO₄ for 2 h.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: α-MEM, α-minimal essential medium; PAS, periodic acid-Schiff; DMs, double minute chromosomes; FCS, fetal calf serum.

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SIGNET RING CELL GASTRIC CARCINOMA CELL LINES

Table 1 Biological properties of HSC-39 and HSC-40A cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth pattern</th>
<th>Doubling time (h)</th>
<th>Growth in agar</th>
<th>Mucus stain</th>
<th>Tumor markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PAS</td>
<td>Alcian blue</td>
</tr>
<tr>
<td>HSC-39</td>
<td>Suspension</td>
<td>28-30</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HSC-40A</td>
<td>Suspension</td>
<td>28-30</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HS-FIB*</td>
<td>Monolayer</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Expression of gastrin and serotonin was performed using the immunohistochemical procedure described previously (15).
* Production of tumor markers and secretin was determined using the culture fluids by standard radioimmunoassay and immunoradiometric assay.
* CEA, carcinoembryonic antigen; SLX, sialyl-Le^-1 antigen; ND, not done.
* A cell line derived from normal human stomach fibroblasts. The cells were used as negative control.

Cellular DNA was digested with appropriate restriction enzymes and processed for Southern blotting hybridization (12). The probe used was a pMC3 plasmid containing a chicken v-myc insert of 1.52 kilobases (13). The β-actin probe was purchased from Oncor, Gaithersburg, MD.

Isolation of RNA and Northern Analysis. Total cellular RNA was extracted from cultured cells using the guanidine isothiocyanate method (14). Cell pellets were resuspended in 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol. The lysate was layered onto 5.7 M CsCl and total cellular RNA was isolated through sedimentation.

For Northern analysis, polyadenylate-selected and total RNA were denatured in 6% formaldehyde and electrophoresed on 1% agarose gels (13). The β-actin probe was purchased from Oncor, Gaithersburg, MD.

RESULTS

Establishment of Two Gastric Carcinoma Cell Lines by Different Methods. Two cell lines were established from the same patient by different methods, one from the ascites (designated HSC-39) and the other from xenotransplanted tumor in an athymic BALB/c nude mouse (designated HSC-40A). Primary cultures were initiated on March 9, 1989. In primary cultures, adherent cells (fibroblastic, mesothelial, and mesenchymal cells) grew rapidly at first and then floating cells were constantly shed from the adherent cell layer into the culture fluid after about 14 days. The first subculture was made about 1 month later by collecting the floating cells. Since then, serial passages have been carried out every 7–10 days.

Another cell line, HSC-40A, was established from the tumor after xenotransplantation of the patient’s ascitic tumor cells in an athymic BALB/c nude mouse. The established cell lines were used for various analyses. Both cell lines were Mycoplasma free as confirmed by electron microscopy and 4,6-diamidino-2-phenylindole dihydrochloride fluorescence staining.

Growth, Morphological, Histochemical, and Immunological Characterization. The biological properties of both cell lines were summarized in Table 1.

The population-doubling time estimated from the growth curve was approximately 28–30 h for both lines. Both lines formed colonies in 0.33% agarose at a cloning efficiency of 0.8–1.0%.

HSC-39 and HSC-40A were similar in morphology (Fig. 1). The cells were round, were floating freely, and tended to aggregate loosely. Single cell suspension was easily obtainable by pipeting. Most of the cultured cells have eccentric nuclei, featuring the signet ring cells which were observed in the original ascitic tumor cells. The cells contained a mucinous substance in the cytoplasm as determined by PAS staining.

HSC-39 and HSC-40A cell lines were investigated for the shedding of tumor-associated antigens in the cell-free culture supernatants. Tumor-associated antigen, CA-125, was not pos-
Table 2  Tumorigenicity of original ascitic gastric carcinoma cells and established cell lines in female athymic BALB/c nude mice

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Pretreatment of recipient</th>
<th>No. of cells injected</th>
<th>Route of injection</th>
<th>Incidence of tumor formation</th>
<th>Growth to $10 \text{ mm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascitic tumor</td>
<td>400 R</td>
<td>$2 \times 10^7$</td>
<td>s.c.</td>
<td>3/4</td>
<td>$66 \pm 9$</td>
</tr>
<tr>
<td>Ascitic tumor</td>
<td>400 R</td>
<td>$2 \times 10^7$</td>
<td>s.c.</td>
<td>0/3</td>
<td>$34 \pm 8$</td>
</tr>
<tr>
<td>HSC-39 (10)$^a$</td>
<td>5 $\times 10^6$</td>
<td>s.c.</td>
<td>5/5</td>
<td>$37 \pm 3$</td>
<td></td>
</tr>
<tr>
<td>HSC-39 (15)</td>
<td>5 $\times 10^6$</td>
<td>i.p.</td>
<td>4/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-40A (15)</td>
<td>5 $\times 10^6$</td>
<td>s.c.</td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-40A (17)</td>
<td>5 $\times 10^6$</td>
<td>i.p.</td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The original ascitic tumor cells were collected and $2 \times 10^7$ viable cells were then injected s.c. into recipients, previously exposed to 4 Gy whole-body X-irradiation.

$^b$ Number of mice with tumors/number of mice given injection.

$^c$ Mean ± SD of the time in days for the s.c. tumors to reach 10 mm mean diameter.

$^d$ Established cell lines derived from different cell culture passages were injected s.c. or i.p.

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**Fig. 2. Ultrastructural characteristics of HSC-39 and HSC-40A cells.**

(A) The cells are spherical. The presence of mucus granules, Golgi complexes, desmosomes (arrows), and tight junctions indicates the mucinous epithelial character. $\times 5600$. Bar, 1 $\mu m$. (B) The cells have numerous secretory granules, and straight microvilli are evident on the surface. $\times 7500$. Bar, 1 $\mu m$. (C) The cells have numerous mucinos granules and secretary granules. $\times 3900$. Bar, 1 $\mu m$.  

**Fig. 3. Histological section of a tumor established by i.p. injection of HSC-40A cells into athymic BALB/c nude mouse.** Proliferation of signet ring cells can be seen among actively growing tumor cells. H & E, $\times 400$.  

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Culture fluids of both cell lines were shown to contain large amounts of carcinoembryonic antigen, CA19-9, and sialyl-Le$^a$-i antigen. Immunohistochemical staining showed the presence of gastrin in these cell lines.

**Electron Microscopic Findings.** Ultrastructural examination demonstrated the presence of desmosomes, confirming the epithelial nature of these cell lines (Fig. 2A, arrows). They were interconnected by numerous desmosomes. Nuclei were round or semiround. Many mitochondria showed pleomorphism. There was an abundance of polysomes. The endoplasmic reticulum and Golgi complexes were well developed. Many mucus granules were also seen in the cytoplasm (Fig. 2, A and C). Secretory granules were numerous and appeared variable in both size and shape. The plasma membrane had numerous straight microvilli (Fig. 2, B and C). At 10 months after the cultivation period, these features were still well maintained and cells were linked by desmosomes and tight junctions.

**Tumor Formation in Athymic Nude Mice.** Tumor nodules developed in athymic BALB/c nude mice at the site of injections following tumor cell graft (Table 2). When cells were injected i.p., ascites was not formed but tumor nodules developed in the peritoneal cavity. Tumor death occurred earlier when the cells were injected i.p. (approximately 80 days) than when they were injected s.c. (>100 days).

Microscopic examination of xenotransplanted tumors showed the active growth of epithelial tumor cells with the presence of signet ring cells (Fig. 3). Many cells were positive...
for PAS staining. Alcian blue staining was negative. Xenotransplanted specimens derived from i.p. or s.c. growth did not differ in their morphological features. None of the xenotransplanted tumors revealed a scirrhous pattern. The xenotransplanted tumors were mostly arranged as groups of tumor cells with poorly developed stromal tissue.

Cytogenetic Findings. Chromosome analysis was carried out on both HSC-39 and HSC-40A at the 30th passage. A modal number of chromosome was 66 in the HSC-39 cells. Many DMs were also found (Fig. 4, top). Analysis of G-banding showed very complex aberrations including rings, two isochromosomes, 5 markers, and 10 derivative chromosomes as presented in Fig. 4, bottom. Loss of chromosome 13 and the Y-chromosome and duplication of the X-chromosome were observed in both cell lines. The representative karyotype was 66,X,+X,−Y,+1,+4,−5,+6,+7,+8,−10,−11,−13,−13, +14,+14,+16,+19,+20,−20,+21,+der(2)(p13,?), +der(2)(p21,?),+der(5)(q22,?),i(9q),i(9q),+der(10)(q10,?),+der(11)(q11,?),+der(12)(q24,?),+der(20)(p10,)?(q13,?),+der(20)(p10,)?(q13,?),+der(22)(p11,?),+der(22)(q22,?),+mar1,+mar2, +mar3,+mar4,+min,+ring,+DMs.

Amplification and Overexpression of the c-myc Gene. We have made an attempt to examine possible changes in oncogenes in HSC-39 and HSC-40A cells because these lines had many DMs. DNAs from the original ascitic tumor cells and two established cell lines were examined with the following probes: Ha-ras, N-ras, K-ras, c-myc, L-myc, erbA, erbB2, myb, src, abl, and mos. Only the v-myc probe detected gene amplification. Fig. 5A shows the results of Southern blot analysis for the c-myc oncogene in HSC-39, HSC-40A, and original ascitic tumor cells. Cellular DNAs extracted from these tumor cells were digested with EcoRI and the filters were hybridized with a 32P-labeled 1.52-kilobase fragment of the chicken v-myc gene. The EcoRI digest of cellular DNA yielded a 14-kilobase band when probed with the v-myc gene. As an internal marker, the human β-actin gene was used (Fig. 5A, bottom).

HSC-39, HSC-40A, and original ascitic tumor cells exhibited a similar level of amplification of the c-myc genes. Comparison of c-myc DNA bands between cultured human stomach fibroblast DNA was performed by serial dilution method (Fig. 5B). The c-myc gene was amplified approximately 16–32-fold.
Fig. 5. Detection and expression of c-myc genes in two established cell lines. (A) High molecular weight DNA (5 μg) from the indicated cells was digested with restriction endonuclease EcoRI and analyzed by the Southern blot hybridization technique using chicken v-myc-specific probe, a 1.52-kilobase (Kb) fragment from pMC3 plasmid DNA. Lane 1, MRC-5 (human diploid fibroblastic cell line); Lane 2, HS-FIB (normal stomach fibroblasts); Lane 3, original ascitic tumor cells; Lane 4, HSC-39 cells; Lane 5, HSC-40A cells. Bottom, rehybridization with 32P-labeled human β-actin complementary DNA as an internal control for the amount of DNA present on the filters. (B) DNA (10 μg) from HSC-39 cells was serially diluted and the intensity of the c-myc bands was compared with that of normal stomach fibroblasts and MRC-5 cell DNA. The degree of dilution is shown above the lanes. (C) Polyadenylate-selected RNA (5 μg) from the indicated cells was analyzed by the Northern blot analysis using human c-myc probe, a 0.4-kilobase fragment from pHSR-1 plasmid DNA (16). Lane 1, HS-FIB; Lane 2, A431 cells; Lane 3, HSC-39 cells; Lane 4, HSC-40A cells (total RNA, 20 μg); Lane 5, HSC-40A (total RNA, 20 μg).

Fig. 5C shows the results of Northern blot analysis for c-myc oncogenes in HSC-39 and HSC-40A cell lines. Expression of the c-myc oncogene was found to be elevated in both cells.

DISCUSSION

We present data of newly established cell lines, HSC-39 and HSC-40A, derived from a signet ring cell gastric carcinoma. Scirrhous gastric carcinoma is morphologically characterized by extensive fibrosis with the occasional presence of poorly differentiated adenocarcinomatous cells with signet ring morphology (2, 3). Human scirrhous gastric carcinoma cells are generally difficult to culture in vitro as permanent cell lines for reasons including overgrowth of fibroblasts, dependence on feeder layers in primary and secondary cultures, and long quiescent periods in culture prior to the active growth. The use of body fluids as a starting material for cell culture provides many technical advantages and has already been used for the establishment of signet ring cell carcinoma of the stomach (4, 17). In addition, we utilized ascitic tumor cells in two ways: (a) ascitic cells were seeded directly into tissue culture media; (b) ascitic cells were injected s.c. into athymic BALB/c nude mice. Athymic BALB/c nude mice have been used for xenotransplantation of human neoplasms, but it has been recognized that scirrhous gastric carcinoma usually resists xenotransplantation.4 However, xenotransplantation in preirradiated athymic BALB/c nude mice was successful, and we were able to obtain a xenotransplantable human gastric carcinoma cell line.

It is important to note that our lines were established from a patient not previously treated with radiation or chemotherapeutic agents. These treatments may result in altering the tumor cell subpopulations in the patient and thus affect the type of cells established from the tumor (18).

The presence of histochemically identifiable PAS-staining cytoplasmic mucin in these cells provides evidence that they are of epithelial origin, showing the same characteristics as the original malignant cells in the patient.

Furthermore, the presence of microvilli, desmosomes, and tight junctions confirmed that these cells are of epithelial cell nature.

The histology of the xenotransplanted tumors resembled that of signet ring cell carcinoma or poorly differentiated adenocarcinoma. However, we were not able to demonstrate the existence of a scirrhous gastric carcinoma pattern in the xenotransplanted tumors.

There are only a few reports on karyotypic changes in human gastric cancer cells examined by the banding technique (19–21). The chromosomal characteristics of HSC-39 and HSC-40A do not resemble those of other gastric carcinoma cell lines, Kato III (4) and M2-Sto-1 (17), which also show cytological features of signet ring tumor cells. Cytogenetic analysis of HSC-39 and HSC-40A cells revealed a narrow range of chromosome number and a marked tendency to triploidy. Analysis of G-banding showed very complex abnormalities including the presence of 5 marker chromosomes. Interestingly, we observed the loss of chromosome 13 which carries the RB gene. This suggests possible loss of function of the RB gene in these cell lines. Motomura et al. (22) reported the loss of alleles at loci on chromosome 13 in human primary gastric cancers. Furthermore, we found loss of the Y-chromosome and duplication of the X-chromosome in our cell lines. Other chromosome aberrations were also observed in both cell lines. However, cytogenetic analysis performed on in vitro-cultivated cell lines may need to compare with that on fresh tumor specimens to clarify possible changes unique to in vitro cultivation of the cells. Nevertheless, our cytogenetic data are compatible with recent reports in which Y-chromosome loss was shown to be common in male human stomach cancers (19–21, 23). The biological significance of Y-chromosome loss in tumor development is unknown. We also found numerous DMs. The presence of DMs usually indicates amplification of genes (24).

In a preliminary screening for gene amplification of cellular protooncogenes, DNA samples from the tumor cells of ascites and two established cell lines were examined with 13 different probes. We detected genomic alterations by using only v-myc
probe and concluded that the c-\textit{myc} gene was amplified 16–32-fold in both cell lines and in the original ascitic tumor cells as well. These data suggest that c-\textit{myc} gene amplification occurred in the original tumor cells and it was not lost during subsequent propagation of the cells both in \textit{vitro} and \textit{in vivo}.

The c-\textit{myc} oncogene is sporadically amplified in various primary tumors and tumor cell lines, but the clinical significance of this phenomenon has not been clarified in these cases (24). Recently, amplification of c-\textit{myc} has been reported in stomach cancer, with frequencies ranging between 7.1 and 11.0% in primary tumor tissues and 18 and 19% in tumor cell lines (25–31). These data suggested that this amplification event could be more frequent in stomach cancer than in other cancers. However, there was no significant correlation between the histological type or growth rate of the tumors and amplification of the c-\textit{myc} oncogene.

In conclusion, we have established an \textit{in vitro} model system of human signet ring cell gastric carcinoma. These cell lines (HSC-39 and HSC-40A) are promising new \textit{in vitro} model systems for studying the poorly understood biology, oncogenic mechanisms, and therapeutic means of signet ring cell gastric carcinoma (or Borrmann’s type IV carcinoma).

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