Unique characteristics of rectal carcinoma cell lines derived from invasive carcinomas in ulcerative colitis patients

Kazuya Yamashita,[1, 2] Shuichi Yasuda,[3] Tatsuru Kuba,[1, 2] Yoshimasa Otani,[4] Mutsunori Fujiwara[5] and Isao Okayasu[1, 2, 6]

[1]Department of Pathology, Kitasato University East Hospital, 2-1-1 Asamizodai, Sagamihara, Kanagawa 228-8520; Departments of [2]Pathology and [4]Surgery and [3]Experimental Animal Center, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555; and [5]Department of Clinical Pathology, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150-8935

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To identify the characteristics of ulcerative colitis (UC)-associated carcinomas, 8 lesions, high-grade dysplasias and invasive carcinomas, were implanted into severely combined immunodeficient (SCID) mice and/or cultured *in vitro*. Intramucosal neoplasias consisting of high-grade dysplasia showed extremely slow proliferation after implantation (2/3 cases) and *in vitro* culture failed (4 cases). However, invasive carcinomas demonstrated rapid growth both after SCID mouse implantation and *in vitro* (4/4 cases). From two cases of invasive carcinomas, 6 cell lines were established, and these are the first to be described in the literature. In addition to variation in immunohistochemically determined phenotypic expression regarding α-fetoprotein, chromogranin A and estrogen receptors, the established cell lines showed varying differentiation (moderately or poorly differentiated adenocarcinoma, adenosquamous carcinoma and poorly differentiated adenocarcinoma with multinuclear giant cells and bone formation). The results are in contrast with findings for sporadic colorectal carcinomas. Although the prevalence of DNA alterations is not frequent, loss of heterozygosity (17p and 18q) and deletion of exons 8 to 11 in DPC-4 were revealed in all of 6 cell lines, suggesting relatively high genetic instability. We found loss or translocation of many chromosomes (#3, 5, 6, 8, 10, 11, 13, 16, 17, 18 and 19) other than chromosomes 1, 5, 8, 11, 13, 17 and 18, which are frequently involved in sporadic colorectal carcinoma cell lines. Thus, the established cell lines may be good models of tumorigenesis and progression in the chronic inflammation-carcinoma sequence. (Cancer Sci 2004; 95: 211–217)

In patients with long-standing ulcerative colitis (UC), colorectal dysplasias and carcinomas frequently develop, and a chronic inflammation-carcinoma sequence has been noted.[1–5] UC-associated tumors have several characteristics, including multiplicity and varying gross appearance of flat, depressed or villous intramucosal lesions with no clear border.[4, 6] Regarding genetic alterations, although *p53* alteration occurs rather often with loss of heterozygosity (LOH), a low prevalence of *APC* and *ras* mutations in UC-associated tumors has been described, along with a high LOH frequency of chromosomes 3 and 18 and *p16*, compared to sporadic colorectal carcinomas.[7–10] Establishment of cell lines of UC-associated tumors should facilitate assessment of differences from the adenoma-carcinoma sequence or the *de novo* cancer pathway. In the present study, UC-associated dysplasias and invasive carcinomas were implanted into severely combined immunodeficient (SCID) mice and cultured *in vitro*. Genetic and histopathological features of the established cell lines were then compared with those of sporadic colorectal carcinomas, previously generated in our laboratory.

Materials and Methods

Source of tumor cells. Neoplastic lesions, including 4 high-grade dysplasias and 4 invasive carcinomas were obtained from surgically resected rectal tissue in 8 cases (21–73 years old; 5 males, 3 females) with UC (Table 1). Total duration of illness was 6 to 18 years. For comparison, cell lines KE-43, KE-43C4 (subclone of KE43) and KE-24, which were established from invasive sporadic carcinoma lesions (ascending and sigmoid colons; 76-year-old male and 54-year-old female, respectively) in our laboratory were used (Table 1). This work was conducted after receiving the standard informed consent from patients and was approved by our Medical School and University Hospital Ethics Committee (No. B01-20).

Implantation of tumor cells into SCID mice and *in vitro* cell culture. After sterilization of surgically obtained tissue with Isogen (Meiji Seika, Tokyo), tumor tissues were cut and used for implantation under the backskin of 6-week-old, female SCID mice (CB-17/ICR-SCID, Nippon Clea, Osaka) and for the primary culture system.

For primary culture, the tumor tissues were minced in ES medium (Nissui, Tokyo) with sharp scissors, treated with 1000 pronase units/ml dispase for 1 h at 37°C, filtered through a 0.1 mm mesh, and centrifuged at 1000 rpm for 10 min. Cell sediments were resuspended in ES medium, containing 15% fetal bovine serum (FBS, Nichirei, Tokyo) and incubated in a culture bottle at 37°C in a CO_2 incubator supplied with humidified 5% CO_2. For subculture, cells were resuspended in 0.02% EDTA, 0.25% trypsin solution and then transferred at a 1:10 dilution into a new culture bottle.

Establishment of cell lines and characterization. With the soft agar colony formation method, 1×10^2, 10^3 and 10^4 cells were incubated in 25 cm^2 dishes containing 0.33% Seaplaq agarose gel for 12 weeks. Then, colonies formed were counted. For transplantation into SCID mice, 2×10^7 incubated carcinoma cells were implanted into the subcutis of the back. Implants were examined for histopathology, ultrastructure and chromosome alteration.

Immunohistochemistry. Immunohistochemical analysis was performed using the standard labeled streptavidin-biotin-peroxidase complex (LSAB kit, DAKO, Carpinteria, CA) method. Briefly, after routine deparaffinization, 4-μm-thick sections were incubated overnight at 4°C with optimal dilutions of primary antibodies. The antibodies used are listed in Table 2. Counterstaining of nuclei was achieved with methyl green. For antigen retrieval, sections were heated in a 10 mmol/liter citrate buffer (pH 6.0) for 20 min, using a microwave oven. Ki-67 labeling indices were determined as percentages by counting at

[6]To whom correspondence should be addressed.
E-mail: isaokaya@med.kitasato-u.ac.jp

least 1000 cells in randomly selected high-power fields (×400). Only nuclei with strong diffuse staining were considered positive.

Electron microscopic analysis. Fresh tumor tissues after implantation and proliferation in SCID mice were fixed in 2.5% glutaraldehyde and postfixed in 2.0% osmium tetroxide. Ultrathin Epon sections were stained with 5% uranyl acetate and 0.5% lead citrate and examined under a transmission electron microscope (Akashi, Nippon Denshi, Tokyo).

Analysis of DNA alterations. Fresh samples of tumor tissues or cancer cells were lysed in SDS-lysis buffer and DNA was extracted with phenol-chloroform following ethanol precipitation for PCR.[11] PCR-SSCP and direct sequencing of *ras* (exons 1, 2), *p53* (exons 5–9),[12] *DCC* (exons 7, 29), *BAX*,[13] *TGF-βRII*[14] and *DPC-4* (exons 8–11) as well as LOH analysis[15] were performed by the reported methods. Five markers (BAT-25, BAT-26, D2S123, D5S346 and D17S250) for MSI in colorectal cancers recommended by NCI[16] were tested in established cell lines. TP-53, D17S578, D17S831 and Mfd-41 were selected for chromosome 17, and D18S34, D18S535 and D18S851 for chromosome 18. A total of 10 markers, D18S51, D5S818, D13S317, D7S820, Amelogenin, D8S1179, D21S11, D3S1358, vWA and FGA, were tested for linkage analysis between the original samples and established cell lines.

Table 1. Analysis of UC-associated lesions

	Dysplasia				Invasive carcinoma (Dukes C type)			
Case No.	1	2	3	4	5	6	7	8
Age (years old) at operation	43	46	71	35	30	73	33	21
Sex	M	M	M	M	M	F	F	F
Duration of UC (years)	18	12	12	16	17	15	11	6
Location of UC	left	total	left	left	total	left	total	total
Primary tumor (original)								
Histology	HGD	HGD	HGD	HGD	Ad.	Ad.	Ad.	Ad.
Differentiation					well	moderately	moderately	poorly
Tumor size	2.0×2.0	3.2×1.8	1.7×1.7	2.0×1.0	9.0×8.0	8.0×7.0	10.5×6.5	7.5×6.0
Implanted tumor in SCID								
Observation time	1 y 6 m	ND	7 m	2 y	6 m	2 m	2 m	2 m
Tumor size	Tumor (–)	ND	0.06 cm^3	0.13 cm^3	2.0 cm^3	3.0 cm^3	5.7 cm^3	7.5 cm^3
In vitro cell culture								
Growth	no	no	no	no	yes	yes	yes	yes
Passage	NA	NA	NA	NA	continued	continued	yes	yes
Cloning	NA	NA	NA	NA	ND	ND	5 clones	NA
Proliferative activity in original								
Ki-67 LI	28.8%	29.4%	20.2%	17.5%	57.9%	52.6%	47.0%	55.4%
Gene analyses								
p53 gene mutation	wild	wild	wild	exon 7 codon 234 TAC/TGC Tyr/Lys	wild	exon 7 codon 244 to 245 6-bp insertion (CGCGGT)	wild	exon 7 codon 248 CGG/TGG Arg/Try
TP-53 locus LOH	stable	stable	LOH	LOH	MSI	LOH	LOH	LOH
DPC4 gene (exons 8 to 11)	wild	wild	wild	wild	wild	wild	Del	Del
LOH score (19 markers)[1)]	0	0	4	2	7	5	10	7

HGD, high-grade dysplasia; Ad., adenocarcinoma; LOH, loss of heterozygosity; ND, not done; LI, labeling index; NA, not available; Del, deletion.
1) LOH analyses markers: D2S123, D3S1358, D5S346, D5S818, D7S820, D8S1179, D13S317, TP53, D17S578, D17S831, D17S831, D18S535, D18S851, D18S51, D18S34, D21S11, vWA, FGA and Amelogenin.

Table 2. Antibodies used in the present study

Antibody	Type	Dilution	Methods		Source
CEA	Mono	1:1000	LSAB		TaKaRa Schuzo, Shiga, Japan
CA19-9	Mono	1:5	LSAB		CIS Bio International, Cedex, France
Chromogranin A	Mono	1:1	LSAB		Lipshaw, PA, USA
AFP	Poly	1:400	LSAB		DAKO, CA, USA
Vimentin	Mono	1:1000	LSAB	AR	DAKO, CA, USA
Placental alkaline phosphatase	Poly	1:80	LSAB		DAKO, CA, USA
Neuron specific enorase	Poly	1:400	LSAB		DAKO, CA, USA
Bcl-2	Mono	1:100	LSAB	AR	DAKO, CA, USA
SCLC	Mono	1:1	LSAB		Zymed, CA, USA
CD44	Mono	1:500	LSAB		Seikagaku Co, Tokyo, Japan
DCC gene products	Mono	1:600	LSAB		Nichirei, Tokyo, Japan
Estrogen receptor	Mono	1:1	LSAB		Nichirei, Tokyo, Japan
c-erbB2	Mono	1:1	LSAB		Nichirei, Tokyo, Japan
c-Myc	Mono	1:1	LSAB	AR	Nichirei, Tokyo, Japan
p53 gene products	Mono	1:500	LSAB	AR	Novocastra, Newcastle upon Tyne, UK.

Poly, polyclonal antibody; Mono, monoclonal antibody; LSAB, streptoavidin-biotin-peroxidase complex method; AR, antigen retrieval method.

Analysis of polymorphic typing was performed using short tandem repeat (STR) markers,[17] an AmpFLSTR Profiler Plus PCR Amplification Kit (PE Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Analysis of chromosome alterations. Fresh original or implanted tumor tissues were examined with the methods described elsewhere.[18] Briefly, after fine mincing without collagenase in a petri dish covered with 3 to 5 ml of RPMI 1640 medium, disaggregated cells were cultured in RPMI 1640 supplemented with 10% FBS plus antibiotics and antimycotics for 1 to 30 days. Metaphase cells arrested by exposure overnight to Colcemid (0.01 mg/ml) were treated with 0.075 *M* KCl hypotonic solution for 30 min at 37°C and then fixed in a 3:1 mixture of methanol:acetic acid. Chromosomes were analyzed using the Q-band technique. Chromosome identification and karyotype designation were performed in accordance with the International System for Human Cytogenetic Nomenclature.[19] After 10 or more metaphase cells were analyzed, chromosome alterations were finally determined.

Statistical analysis. Statistical comparisons between two groups were performed with the nonparametric Mann Whitney *U* test.

Results

Primary tumors in UC for analysis. Clinicopathological and genetic features of the primary tumors used for implantation and/or *in vitro* cell culture are summarized in Table 1. Ki-67 labeling indices were significantly lower ($P<0.0209$) in high-grade dysplasias (mean±SD, 24.0±6.0%) than in invasive carcinomas (53.2±4.7%). *K-ras* gene was wild type in all of 8 tumors, but the *p53* gene (exon 7) was mutant in 3. *DPC4/Smad* (exons 8–11) was deleted in 2 invasive carcinomas. LOH for TP-53 was positive in 5 tumors and microsatellite instability was evident in one. TGF-βRII was microsatellite-stable in all of 8 tumors. LOH in other markers was more frequent in invasive carcinoma (mean±SD, 7.3±2.1 in 19 markers) than in dysplasia (1.5±1.9), the difference being significant ($P=0.0209$).

Implantation of UC-associated tumors into SCID mice and *in vitro* cell culture. After implantation, tumors were detected with 2 high-grade dysplasias and 4 invasive carcinomas (Dukes C) and lacking in 1 case of high-grade dysplasia (1 case, not implanted), after 2–24 months. However, the growth of implanted tumors in 2 cases of high-grade dysplasia was too slow for gross identification (7 and 24 months; tumor size, 0.06 and 0.13 cm^3). Rapid growth with 4 cases of invasive carcinomas was confirmed in a relatively short time (2, 2, 2 and 6 months; tumor size, 2.0, 3.0, 5.7 and 7.5 cm^3) after implantation. The implanted tumors in these cases were further transplanted into new SCID mice.

With *in vitro* cell culture, cell growth was not evident with any of the 4 cases of high-grade dysplasia. In contrast, carcinoma cells from all invasive carcinomas proliferated and could be serially subcultured. Further, carcinoma cell lines were established in cases 7 and 8 after serial subculture for 3 and 4 years, respectively.

Carcinoma cell lines (Table 3). In case 7, cancer cells adhered to the surface of the culture bottle 24 h after primary culture, and cell proliferation was confirmed 7 days later. Implantation of primary culture cells in SCID mice resulted in tumors after 8 weeks (tumor take, 4/4, 100%). Carcinoma cell proliferation became stable after 7 passages. In the 10th passage culture, 5 clones (UCCA-21, 22, 23, 24, 25) were separated by the single cell cloning method with soft agar colony formation.

In case 8, primary culture failed to generate carcinoma cell proliferation due to bacterial and fungal infection. However, growth of implants was detected in SCID mice 8 weeks after implantation (tumor take, 2/4, 50%), and *in vitro* culture of the lesions resulted in stable growth of the UCCA-3 line. Passage of carcinoma cells to SCID mice was also stable.

Doubling times of incubated carcinoma cells were assessed by the trypan blue dye exclusion method, counting every day for 14 days after incubation of 2×10^6 cells. Doubling times were 63.6, 43.8, 44.4, 37.3, 40.5 and 56.9 h for UCCA-21, 22, 23, 24, 25 and UCCA-3, respectively.

Characterization of established cell lines (Tables 3–5)

Histological features. Examination of histologic sections of implanted tumors in SCID mice revealed adenocarcinomas, having tubular or microtubular structures, in the UCCA-21 (moderately differentiated adenocarcinoma), 22 (poorly differentiated adenocarcinoma), 23 (adenosquamous carcinoma), 24 (poorly differentiated adenocarcinoma) and 25 (adenosquamous

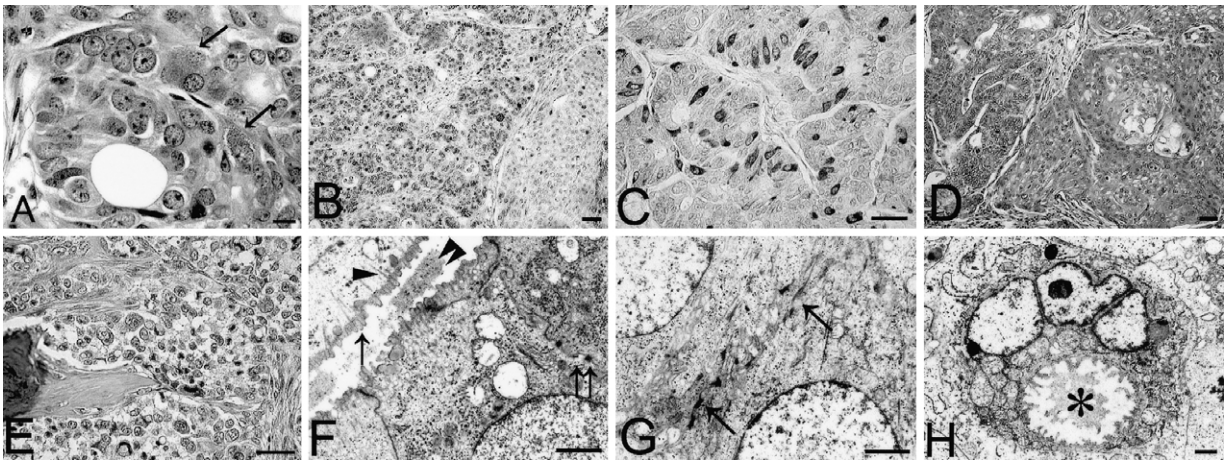

Fig. 1. Morphological features of implanted cell lines on hematoxylin-eosin staining (A, D and E), immunostaining (B and C) and electron microscopic sections (F–H). A, Paneth cells (arrows) in an implanted tumor of the UCCA-21 cell line. B, p53-positive cells in implanted UCCA-21 cells. C, Scattered distribution of chromogranin A-positive cells in implanted UCCA-21 cells. D, Adenosquamous carcinoma with keratinization in implanted UCCA-25 cells. E, Implanted UCCA-3 cells forming giant cells and bone (Bar represents 50 μm). F, Adenocarcinoma differentiation of UCCA-21 including rootlets (arrowhead), microvilli (arrow), desmosome structures (double arrows) and a glycocalceal body (double arrowheads). G, Tonofilament-desmosome complex (arrows) indicating keratinization in UCCA-25. H, Intracytoplasmic lumen (asterisk) indicating adenocarcinoma differentiation in UCCA-3 (Bar represents 0.5 μm).

carcinoma, focally with keratinization). In UCCA-21, differentiation into Paneth cells, enterochromaffin cells and goblet cells was seen. UCCA-3 exhibited features of poorly differentiated adenocarcinoma, with mixed giant cells and bone formation, microtubular structures and medullary growth, also found partly in the primary tumor (case 8) (Fig. 1) (Table 3).

From sporadic colorectal carcinomas, all of 3 cell lines implanted in SCID mice were well or poorly differentiated adenocarcinomas without squamous cell differentiation (KE-24, KE-43 and KE-43C4).

Immunohistochemistry. Diffuse or scattered expression of CEA, CA19-9, α-fetoprotein, CD44 and p53 was common in all 5 cell lines derived from case 7 (Fig. 1). Scattered expression of chromogranin A (Fig. 1), estrogen receptors and DCC was seen only in UCCA-21. Bcl-2 expression was evident in UCCA-21 and 25 but not in the original tumor. Weak expression of c-myc was seen in all cell lines except for UCCA-21. Diffuse or scattered expression of CEA, CA19-9, chromogranin A, c-myc and p53 was found for UCCA-3 (Fig. 1) (Table 3).

In comparison, cell lines from sporadic colorectal carcinomas had no expression of the above markers, except for diffuse or strong expression of CEA, CA19-9 and c-Myc.

Ultrastructural features. Ultrastructurally, desmosome structures were confirmed in all cell lines, indicating an epithelial cell origin. UCCA-21, 22, 23, 24, 25 and UCCA-3 (Fig. 1) all showed adenocarcinoma differentiation, including rootlets, microvilli, glycocalceal bodies and mucin as typical features. Glycogen, tonofilaments and tonofilament-desmosome complexes (only in UCCA-25) were also seen in UCCA-23 and 25, as squamous cell features.

DNA alterations. Basically, DNA alterations in established cell lines were the same as in the original tumors (UCCA-21-25 from case 7 and UCCA-3 from case 8) (Table 4). LOH in 17p13 (TP-53) was seen in all the cell lines (6/6) established from UC-associated tumors, as well as the primaries. LOH in D17S831 was detected in cell lines, UCCA-21-25. LOH in 18q21 (D18S535, D18S851 and D18S51) was also often found in cell lines from UC-associated tumors. LOH in other markers was more frequent in UC-associated carcinoma cell lines (mean±SD, 2.0±0.0 in 9 markers) than in sporadic colorectal carcinoma cell lines (0.0±0.0), the difference being significant (P=0.0201). A point mutation in *p53* exon 7 (codon 248 CGG/TGG) was evident in UCCA-3, as well as in the original tumor in case 8. One base deletion of *BAX* was revealed only in UCCA-21. A *DPC-4* gene deletion (exons 8 to 11) was found in all 6 cell lines (Table 4). *Ki-ras* mutations were not found in any primary UC-associated tumors and cell lines, although all cell lines from sporadic colon cancers showed mutations.

Chromosome alterations. All of the established cell lines exhibited human chromosome types. The chromosomal make-up of the primary and a metastatic tumor in the lymph nodes in case 7 is shown in Table 5. Common alterations were der(3)t(3;8)(p12;q11), −5, −6, del(9)(p12), −18, −19, +mar1, +mar2, +mar3, +mar4, indicating an intimate relation between the lesions. UCCA-21, 22, 23, 24, 25 obtained by cloning of the metastatic tumor had common features as follows: chromosome number, 41−44; sex chromosome, XX; common alterations, add(3)(p25), der(3)t(3;8)(p2;q11), −4, −5, −6, −8, del(9)(p12), −10, add(11)(q23), −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4, showing a high similarity to those of the original metastatic tumor in the lymph node. With clone UCCA-21, the chromosome pattern was the same as for clone UCCA-22. Chromosome-13 was seen in clones UCCA-21, -22, -23, -24. The add(15)(q26) was detected only in UCCA-25.

In case 8, chromosome analysis of the primary tumor was not performed because of bacterial contamination at surgery. The implanted tumor in SCID mouse had chromosome number, 40; sex chromosome, XX;−4, add(4)(p16), del(8)p11, add(11)(p11), −14, −15, −16, −17, −18, −22, +r1. The established cell line (Fig. 1B), UCCA-3, had the same alteration, add(11)(p11), as the implanted tumor.

Table 3. Histological characteristics of cell lines established from UC-associated carcinomas and comparison with sporadic colorectal carcinomas

	UC-associated carcinomas						Sporadic colorectal carcinomas		
Histology Differentiation	(Case 7) Adenocarcinoma moderately					(Case 8) Adenocarcinoma poorly	Adenocarcinoma poorly	Adenocarcinoma well	
Cell line name	UCCA-21	UCCA-22	UCCA-23	UCCA-24	UCCA-25	UCCA-3	KE-24	KE-43	KE-43C4
Histology Differentiation	Ad. moderately	Ad. poorly	Adeno-squamous	Ad. poorly	Adeno-squamous	Ad. poorly	Ad. poorly	Ad. well	Ad. poorly
Doubling time	63.6	43.8	44.4	37.3	40.5	56.9	32.4	33.6	63.6
Chromosome (mode)	41–42, XX	41, XX	41–42, XX	40–43, XX	41–44, XX	69–74, XX	46, XY	42, XX	41, XX
PAS staining	+	+	+	+	+	+	+	+	+
Alcian blue staining	+	−	−	−	−	+	+	+	+
CEA	diffuse	diffuse	diffuse	diffuse	diffuse	diffuse, weak	diffuse	diffuse	diffuse
CA19-9	focal, weak	focal, weak	focal, weak	focal, weak	focal, weak	diffuse, weak	diffuse	diffuse	diffuse
α-Fetoprotein	scattered	diffuse	diffuse	diffuse, weak	diffuse	—	—	—	—
Chromogranin A	scattered	—	—	—	—	scattered	—	—	—
Vimentin	scattered	scattered	scattered	—	scattered	—	—	—	—
Bcl-2 (original, negative)	scattered	—	—	—	scattered	—	—	—	—
CD44	scattered	diffuse	diffuse	diffuse	focal	—	—	—	—
Estrogen receptor	diffuse	—	—	—	—	—	—	—	—
DCC	focal	—	—	—	—	—	—	—	—
c-Myc	—	weak	weak	weak	weak	strong	strong	—	strong
p53	diffuse	diffuse	diffuse	diffuse	diffuse	diffuse	scattered	—	—

Ad., adenocarcinoma.

Table 4. Genetic characteristics of cell lines established from UC-associated carcinomas and comparison with sporadic colorectal carcinomas

	UC-associated carcinomas						Sporadic colorectal carcinomas		
	UCCA-21	UCCA-22	UCCA-23	UCCA-24	UCCA-25	UCCA-3	KE-24	KE-43	KE-43C4
Ki-RAS exons 1 to 2	wild	wild	wild	wild	wild	wild	codon 61 CAA/CGA	codons 12 GGT/GTT	
Ha-RAS exons 1 to 2	wild	wild	wild	wild	wild	wild	wild	codon 67 ATG/ACG	
p53 exons 5 to 9	wild	wild	wild	wild	wild	codon 248 CGG/TGG	wild	wild	
DPC-4 exons 8 to 11	exons 8–11 del.					exons 8–11 del.	wild	exons 8–11 del.	
DCC exon P & 29	wild	wild	wild	wild	wild	wild	wild	del./del.	
BAX gene	1 oligo del.	stable	stable	stable	stable	stable	1 oligo del.	stable	stable
TGFβRII	stable	stable	stable	stable	stable	stable	1 oligo add.	stable	stable
BAT-25*	MSI	stable	stable	stable	stable	MSI	MSI	stable	stable
BAT-26*	stable	stable	stable	stable	stable	stable	stable	stable	stable
BAT-40	stable	stable	stable	stable	stable	stable	del.	stable	stable
D2S123*	stable	stable	LOH	stable	MSI	stable	stable	stable	stable
D5S346*	stable	stable	MSI	stable	stable	stable	LOH	stable	stable
D17S250*	stable	stable	stable	MSI	MSI	stable	stable	stable	stable
TP-53	LOH	LOH	LOH	LOH	LOH	LOH	MSI	stable	stable
D17S578	stable	stable	stable	stable	stable	stable	MSI	stable	stable
D17S831	LOH	LOH	LOH	LOH	LOH	stable	stable	stable	stable
D18S535	LOH	LOH	LOH	LOH	LOH	LOH	LOH	LOH	
D18S851	stable	LOH	LOH	LOH	LOH	LOH	MSI	del.	
D18S51	del.	LOH	LOH	LOH	LOH	LOH	stable	del.	
D5S818	LOH	LOH	LOH	LOH	LOH, MIS	stable	stable	stable	stable
D13S317	LOH	LOH	LOH	LOH	LOH	stable	stable	MSI	MSI
LOH score (9 markers)[1)]	2	2	2	2	2	2	0	0	0

oligo, oligonucleotide; del., deletion; add., added; MSI, microsatellite instability; LOH, loss of heterozygosity.
* NCI recommended markers: BAT-25, BAT-26, D2S123, D5S346, D17S250.
1) LOH analyses markers: Mfd-41, D18S34, D7S820, D8S1179, D21S11, D3S1358, vWA, FGA and Amelogenin.

Table 5. Chromosome analysis of cell lines established from UC-associated carcinomas

Case 7				
Primary tumor		39,	X, −X,	der (3)t(3;8)(p12; q11), −5, −6, −8, del(9)(p12), −10, add(11)(q23), −12, −13, i(13)(q10), add(14)(p13), −16, −17, −17, −18, −19, +mar1, +mar2, +mar3, +mar4, +mar5
Metastatic timor		42,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, +7, −8, del(9)(p12), −10, add(11)(q23), −13, −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4, +mar5
Cell line				
	UCCA 21	41–42,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, −8, del(9)(p12), −10, add(11)(q23), −13, −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4
	UCCA 22	41,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, −8, del(9)(p12), −10, add(11)(q23), −13, −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4
	UCCA 23	41–42,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, −8, del(9)(p12), −10, add(11)(q23), −13, −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4[cp6]/41, idem, −13, +mar5[cp4]
	UCCA 24	40–43,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, −8, del(9)(p12), −10, add(11)(q23), −13, −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4[cp6]/41–42, idem, add(11)(p15), +21[cp4]
	UCCA 25	41–44,	XX,	add(3)(p25), der(3)t(3;8)(p12;q11), −4, −5, −6, +7, −8, del(9)(p12), −10, add(11)(q23), add(15)(q26), −16, −18, −19, +20, −22, +mar1, +mar2, +mar3, +mar4
Case 8				
Implanted tumor		40,	XX,	−4, add(4)(p16), del(8)(p11), add(11)(p11), −14, −15, −16, −17, −18, −22, +r 1[10]
Cell line				
	UCCA 3	69–74,	XX,	del(1)(p36), del(3)(p13), add(6)(q27), add(11)(p11), add(14)(p11), add(15)(p11), +mar1 x2, +mar2 x2, +mar3 x2, +mar4 x2, +3–9mar, inc[cp10]
Sporadic colorectal carcinomas				
	KE-24	46,	XY,	t(1; ?)(q42;?), del(6)(p23), add(14)(q32)
		47,	XY,	t(1; ?)(q42;?), del(6)(p23), +7, add(14)(q32)
		46,	XY,	add(1)(q42), t(6; 14)(p23; q32)
	KE-43	41,	X, −X,	add(1)(p36), +2, −3, −4, +6, i(6)(p25), add(6)(q23), −8, −10, add(11)(q25), del(12)(p12), −13, −15, -17, −18, −21, −22, −22, +mar1, +mar2, +mar3, +mar4, +mar5
	KE43C4	42,	XX,	add(1)(p36), del(3)(q25), −4, del(5)(q35), add(6)(q23), −10, −11, del(12)(p12), −13, add(14)(q32), −15, −16, −17, −18, −18, +20, −22, +mar1, +mar2, +mar3, +mar4, +mar5

Discussion

Tumor growth in SCID mice was confirmed for 6 of 7 UC-associated neoplasms. Invasive carcinomas in all of 4 cases showed rapid growth both in SCID mice and *in vitro* culture, in line with the aggressive infiltration of the original tumors. However, the growth of high-grade dysplasias in SCID mice was extremely slow in 2 cases, indicating very slow proliferative activity for intramucosal neoplastic lesions. Clinically, all patients with high-grade dysplasia were alive 8 months to 7 years after surgical resection. Conversely, the patients died within 1 year after surgery in 3 of the 4 cases with invasive carcinoma. Therefore, growth features in SCID mice and *in vitro* culture coincide with those of the original tumors (Ki-67 labeling indices).

Histological features of the implanted cell lines were moderate or poor differentiation, with adenosquamous elements in some cases and bone formation. In particular, cell lines from UC-associated tumors expressed proteins, such as α-fetoprotein, chromogranin A, CD44 and estrogen receptor (UCCA-21) as well as CEA and CA19-9, generally not found in cell lines from sporadic colorectal carcinomas. Consequently, UC-associated carcinomas may have greater potential for anomalous differentiation. Several cases of teratoma,[20] carcinoid [21] and hepatoid adenocarcinoma [22] have been described in UC cases, again suggesting a potential for differentiation towards various kinds of cells.

Most established cell lines in previous reports exhibited similar histopathological features to the original tumors, like our cell lines from sporadic colorectal tumors. In the present study, the established cell lines showed considerable differences from the original UC-associated carcinomas. The possibility of contamination with other cell lines or transformed cells derived from SCID mice could be ruled out as follows. With polymorphic typing, genotypes of 10 markers for linkage analysis demonstrated complete identity between established cell lines and original tumors in cases 7 and 8. Frequent LOH in established cell lines was confirmed in 5 (D18S51, D5S818, D13S317, D3S1358 and vWA) of 10 markers, identical to the original tumors.

Further, characteristic alterations of chromosomes were confirmed in primary tumors, implanted SCID mice and cell lines. These chromosome alteration patterns were rather different from the frequent involvement of chromosomes 1, 5, 8, 11, 13, 17 and 18 in reported cell lines of sporadic colorectal carcinomas, where our sporadic colon cancer cell lines KE-43, KE-43C4 and KE-24 also showed alterations (Table 5).[23, 24]

There have been many reports indicating relatively low mutation frequencies in UC-associated tumors: 6% for *APC*, 3 to 50% for *ras*, 35 to 50% for *p53* and 25% for *DPC-4*.[25–32] Positive results with LOH analysis were 33 to 43% for *APC* (5q), 33 to 85% for *p53* (17p) and 78% for *DPC-4* (18q).[33–35] Fogt *et al*., 1998[8] proposed that LOH of *p16* (9q) and *p53* (17p), related to DNA repair systems, might trigger further LOH of *APC* (5q) and *DCC* (18q), as characteristic features of UC-associated tumorigenesis. In the present study, while mutations or LOH of *ras*, *DCC*, *BAX* and *TGF-βRII*[36] were low or absent, LOH of 17p and 18q were frequent in our cell lines of UC-associated cancers, consistent with the original tumors, and in line with high epithelial and stromal genetic instability of chromosome 17 in UC-associated carcinogenesis in our recent report.[37] In particular, it is interesting that homo-deletion of the *DPC-4* gene was seen in all of 6 established cell lines, suggesting dysregulation of the TGFβ-Smad4/DPC-4 signaling system which regulates cell proliferation.[28, 36, 38, 39] Therefore, alterations of 17p and 18q genes may be strongly related to tumor-development and progression in UC-associated tumorigenesis. In addition, LOH in D5S818, D13S317 and other markers was relatively frequently seen in UC-associated carcinoma cell lines, compared to sporadic colorectal carcinoma cell lines, indicating more genome-wide alterations in the former. The above genetic alterations might be related to UC-associated carcinogenesis, but further study is needed to establish this.

In conclusion, our novel cell lines, established from UC-associated tumors, may be good models for analysis of tumorigenesis and progression in the chronic inflammation-carcinoma sequence.

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