Establishment of Human Colon Cancer Cell Lines from Fresh Tumors versus Xenografts: Comparison of Success Rate and Cell Line Features

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Abstract

Obtaining representative human colon cancer cell lines from fresh tumors is technically difficult. Using 32 tumor fragments from patients with colon cancer, the present study shows that prior xenograft leads to more efficient cell line establishment compared with direct establishment from fresh tumors ($P < 0.05$). From 26 tumor specimens, we successfully established 20 tumor xenografts in *nude* mice (77%); among 19 of these xenografts, 9 (47%) led to cell lines, including four from liver metastases. Only 3 of 31 tumor specimens (9.7%) grew immediately *in vitro*, and all were derived from primary tumors. To compare major phenotypic and genotypic characteristics of human colon cancer cell lines derived from the same tumor fragment using two protocols, the two pairs of cell lines obtained from 2 of 32 tumor fragments were extensively studied. They displayed similar morphology and were able to form compact spheroids. Chemosensitivity to 5-fluorouracil, CPT11, and L-OHP differed between cell lines obtained from patient tumors and those derived from xenografts. Matched cell lines shared a common core of karyotype alterations and distinctive additional chromosomal aberrations. Expression levels of genes selected for their role in oncogenesis evaluated by real-time quantitative PCR were found to be statistically correlated whatever the *in vitro* culture model used. In conclusion, xenotransplantation in mice of tumor fragments before establishment of cell lines enables generation of more novel human cancer cell lines for investigation of colon cancer cell biology, opening up the opportunity of reproducing the diversity of this disease. [Cancer Res 2007;67(1):398–407]

Introduction

Colorectal cancer accounts for 10% to 15% of all cancers and is the second leading cause of cancer-related death in industrialized countries. Despite significant progress in identifying accumulated genetic abnormalities contributing to the malignant phenotype of colon cancer cells (1, 2), a more detailed understanding of colon carcinoma generation and progression is needed. Likewise, the treatment of colorectal cancer has undergone substantial improvements in the last 10 to 15 years in terms of screening, surgical management of resectable disease, and adjuvant chemotherapy (3). Nevertheless, 5-year survival for all patients has improved from only 50% to 62% during the last 25 years (4, 5).

Colon cancer is recognized as a heterogeneous disease that may be amenable to different adjuvant treatments, depending on its molecular characteristics (6). Two major genetic pathways, characterized by microsatellite and chromosomal instability, have been identified in the development of colon cancer (7, 8). In addition to this disease-related heterogeneity, intrinsic tumor heterogeneity is observed, which is a landmark of colon cancer. Gain and loss of chromosomes in the malignant cell population is considered a process of diversification that leads to survival of the fittest clones (9, 10). Although extensive genotypic and phenotypic heterogeneity is recognized as a major trait of cancer cell populations, this heterogeneity could be rapidly masked by the expansion of a limited number of clones.

Studies using low-passage human cancer cell lines remain essential to gain further insight into oncogenic mechanisms and to test new adjuvant chemotherapy strategies in colon cancer. However, technical difficulties continue to be encountered when generating this biological material. The present study was undertaken in an attempt to increase the success rate of cell line establishment by engrafting tumor fragments in immunocompromised mice before the *in vitro* culture step. This study is also the first to compare two pairs of novel colon carcinoma cell lines obtained either directly from two colon tumor fragments or after corresponding established xenograft.

Materials and Methods

Patient tumors. Colon cancer samples were obtained from the Institut Gustave Roussy in accordance with protocols approved by the local

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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ethical committee. Surgical specimen size had to be sufficiently large to be able to undergo both *in vitro* treatment and xenografting. Consequently, all patients included in this study were at an advanced stage of colon cancer, requiring palliative colectomy. Tumor material not required for histopathologic diagnosis was both snap-frozen in liquid nitrogen and placed in "collecting medium" (DMEM supplemented with 10 mmol/L HEPES, 4.5 g/L glucose, 1 mmol/L pyruvate sodium, 200 units/mL penicillin, 200 μg/mL streptomycin, 200 μg/mL gentamicin, 5 μg/mL ciprofloxacine, 20 μg/mL metronidazole, 25 μg/mL vancomycin, and 2.5 μg/mL fungizone) for further establishment of cell lines or xenografts as follows.

Preparation of xenografts. Five-week-old Swiss *nu/nu* (*nude*) male mice were used as xenograft recipients for human tumor fragments. They were bred in the animal facilities of the Institut Curie and maintained in specified pathogen-free conditions. Their care and housing were in accordance with institutional guidelines as put forth by the French Ethical Committee (Agreement B75-05-18, France) under the supervision of an authorized investigator (M.F. Poupon). Tumor s.c. implantation procedures were done as previously described (11).

Establishment of cell lines and cell cultures. Tumor samples from patients or xenografts underwent similar *in vitro* culture protocols. Tumor tissue was finely minced and, after extensively washing with culture medium, transferred into a 25-cm^2 culture flask at 37°C, with 8% CO_2. The culture medium used consisted of collecting medium supplemented with 10% decomplemented FCS. After passage 8, cells were cultured in culture medium devoid of gentamicin, vancomycin, and fungizone. Controlled trypsinizations were done to preferentially remove the contaminating fibroblasts. The cells were routinely passed once a week, and the medium was changed twice in between. All cell lines were initially found contaminated (Mycoplasma PCR detection kit VenorGeM, Minerva Bioloabs, Biovalley, Marne-la-Vallée, France) and were thus treated with *Mycoplasma* elimination reagent (Mynox, Minerva Biolabs). DNA fingerprint experiments were done with all cell lines (data are available in Supplementary Data).

Three-dimensional multicellular spheroids were prepared by the liquid overlay technique (12). In brief, tissue culture microplates were coated with 75 μL of 1% agarose in water. Tumor cells from established cell lines grown as a two-dimensional monolayer were resuspended with trypsin, and 5×10^3 cells per microwell were seeded in 150 μL of culture medium to obtain a single spheroid per well after 4 days.

Morphologic studies. The surgical and xenograft tissue samples were fixed in 10% formaldehyde, paraffin embedded, and stained with H&E and safran. Cell lines were cultured as a monolayer on Lab-Tek chamber slides. They were fixed for 20 min at −20°C in 50% acetone/50% methanol. Spheroids were included in a Shandon cryomatrix (Thermo Electroncorporation, Saint Herblay, France) and placed in liquid nitrogen before processing with a cryostat to yield 7-μm sections. These sections were air-dried for 2 h and then fixed in acetone for 10 min. H&S staining was then done on fixed cells.

Growth kinetics. Growth kinetics was determined by trypsinizing the cultures in triplicate and resuspending the cells in medium. The viable cells were counted using a light microscope by trypan blue dye exclusion test at days 3, 5, 7, and 10. For propidium iodide staining, exponentially growing cells were harvested using trypsin, resuspended in 10% FCS-medium, and washed twice in PBS. Cells were fixed with cold 70% ethanol and stained with propidium iodide (60 μg/mL) containing RNase A (20 μg/mL). Measurement of at least 10,000 nuclei was done using an EPICS XL cytometer (Coultronics, Coulter, Villepinte, France). For Ki67 staining, cell lines were cultured as a monolayer on Lab-Tek chamber slides, fixed using 5% formaldehyde/2% sucrose in PBS for 10 min, and permeabilized with 0.5% NP40, 10% glucose and decomplemented FCS 1% in PBS for 5 min. Ki67 immunostaining was carried out with the Ki-S5 clone (DAKO, Trappes, France). Bound antibody was detected using the LSAB2 System, AP (DAKO) according to the manufacturer's instructions. The Ki67 score was defined as the average number of cells with nuclear staining divided by the total number of cells counted. Two hundred tumor cells were counted, and the process was repeated thrice. Data were compared using the paired Student's *t* test.

Cytotoxic assays. Cells were seeded into 96-well microtiter plates at 5,000 per well in 150 μL. At that density, the control cells were still exponentially growing at the assay end point. Three days after plating, 50 μL of supernatant were removed, and 100 μL of 5-fluorouracil (5-FU; ICN, Orsay, France), CPT11 (Aventis, Vitry-sur-Seine, France), or L-OHP (Sanofi-Synthelabo, Notre-Dame de Bondeville, France) were added to triplicate wells. After 3 days of exposure, the viable cell number in treated versus control wells was estimated by WST assay (Roche Diagnostics, Meylan, France). Drug effects were compared at the level of 50% inhibition (IC_{50}) in comparison with control. Data were reported as means ± SE and were compared using the unpaired Student's *t* test.

Determination of mRNA levels using real-time PCR. Frozen tissue specimens from the patient's resected tumor and the derived xenograft were serially cut. The first 5-μm section was stained with H&E for histologic analysis to determine the ratio of viable cancer cells to normal contaminating cells. Steps of RNA extraction to reverse transcription-PCR were conducted as previously described (13). Standard curves were established using cDNA of five serial dilutions of the universal human reference RNA (20–0.032 ng per tube) from Stratagene (La Jolla, CA). Each PCR run, carried out in duplicate, included the five points of the standard curve and a no-template control. Three housekeeping genes were studied (*RPLPO, TBP*, and *ACTB*) in addition to 66 candidate genes chosen for their implication in key alterations governing oncogenesis (Table 1). To quantify the gene expression level in each sample, the comparative threshold cycle (C_T) method was used according to the manufacturer's instructions. A C_T of 40 means no gene amplification and was taken into account in addition to quantitative levels of mRNA within the standard curve values. Within tissue and cell samples, the geNorm approach led to identification of *ACTB* as the most stably expressed gene among the 69 genes studied (http://www.wzw.tum.de/gene-quantification/; ref. 14). Finally, each gene level was then normalized by dividing its level by the level of the reference gene *ACTB*. StatView 5.0 software was used for statistical analysis. Pearson correlation coefficients were calculated between gene expression data sets of candidate genes from various samples.

Cytogenetic analyses. Cytogenetic analyses were done using R-banding on exponentially grown monolayer cell cultures. Cell cultures, chromosome preparations, and R-banding were done according to the usual methods (15).

Results

Establishment of novel cancer cell lines from 32 patient tumors. By combining *in vitro* and *in vivo* approaches, we attempted to increase the success rate for establishing novel permanent human colon cancer cell lines. Thirty-two colon cancers originated from primary tumors and/or metastases were included in this cell line establishment study. Table 2 shows a summarized description of clinicopathologic data. Surgical specimens were divided into two pieces: one was used for direct *in vitro* establishment of the cell line, whereas the other was used for grafting in mice. When serially transplantable xenografts were established, tumor tissue derived from xenotransplant was used for the *in vitro* cell line establishment assay. Thirty-one of 32 surgical specimens were used for direct *in vitro* establishment protocol, and 26 were grafted s.c. into nude mice. This step of prior xenografting was shown here to lead to more efficient *in vitro* cell line establishment ($P < 0.05$): we successfully established 20 tumor xenografts in nude mice from among 26 tumor specimens (77%), and among 19 of these xenografts, 9 (47%) led to established cell lines. In contrast, only 3 of 31 (9.7%) grew immediately in cell culture.

Association with clinicopathologic data. It is noteworthy that the three cell lines obtained directly from fresh patient

Table 1. List of genes selected for their implication in oncogenesis and studied using real-time reverse transcription-PCR

Gene	Encoded protein	Chromosomal location
AKT1	RAC	14q32.32
AKT2	AKT2	19q13.1-q13.2
ANGPT1	Angiopoietin 1	8q22.3-q23
ANGPT2	Angiopoietin 2	8p23.1
BAD	BAD	11q13.1
BAX	BAX	19q13.3-q13.4
BCL2	BCL2	18q21.3
BIRC5	Survivin	17q25
BRMS1	BRMS1	11q13-q13.2
CASP9	Caspase-9	1p36.3-p36.1
CAT	Catalase	11p13
CCNA2	Cyclin A2	4q25-q31
CCNB1	Cyclin B1	5q12
CCND1	Cyclin D1	11q13
CCNE1	Cyclin E1	19q12
CCNE2	Cyclin E2	8q22.1
CCNG2	Cyclin G2	4q21.1
CD82	CD82	11p11.2
CDC2	CDK1	10q21.1
CDC42	CDC42	1p36.1
CDH1	E-cadherin	16q22.1
CDK4	CDK4	12q14
CDKN1A	p21	6p21.2
CDKN2A	p16	9p21
CDKN2B	p15	9p21
CGB	HCG beta	19q13.32
EGFR	EGF-R	7p12
ERBB2	c-erbB2	17q21.1
FAS	Fas	10q24.1
FASLG	FasL	1q23
FOS	c-*fos*	14q24.3
GPR54	KiSS-1R	19q13.3
HPSE	Heparanase	4q21.3
IGF1	IGF1	12q22-q23
IGF2	IGF2	11p15.5
ITGAE	Integrin αE	17p13
JUN	c-jun	1p32-p31
KRAS	Ki-ras	12p12.1
LAMA5	Laminin-α5	20q13.2-q13.3
MAP2K4	MAP2K4	17p11.2
MDM2	MDM2	12q14.3-15
MMP2	MMP2	16q13-q21
MMP9	MMP9	20q11.2-13.1
MYC	c-*myc*	8q24.12-q24.13
NME1	NM23	17q21.3
PIK3CA	PI3K	3q26.3
PLAU	Urokinase	10q24
PLAUR	uPAR	19q13
PTEN	PTEN	10q23.3
RAGE	RAGE 1	14q32
RHOC	RhoC	1p13.1
SERPINB5	Maspin	18q21.3
SNAI1	Snail	20q13.1-q13.2
SOD1	SOD1	21q22.11
TGFA	TGFα	2p13
TGFB1	TGFβ	19q13.1
TIMP1	TIMP1	Xp11.3-p11.23
TIMP2	TIMP2	17q25
TIMP3	TIMP3	22q12.3
TIMP4	TIMP4	3p25
TP53	p53	17p13.1
VEGF	VEGFA	6p12
VEGFB	VEGFB	11q13
VEGFC	VEGFC	4q34.1-q34.3
VEGFD	VEGFD	Xp22.31
VIM	vimentin	10p13

tumors all originated from primary tumors. Clinicopathologic characteristics of tumors leading to or not leading to cell lines and/or xenografts were compared and analyzed using the χ^2 test or unpaired Fisher's test. Only the origin of the tumor (primary tumor versus metastases) was statistically related to the success rate of cell line establishment from fresh tumors (cell lines obtained directly from surgery specimens: 3 of 11 primary tumors and none of 20 metastases, $P < 0.05$). In contrast, cell line establishment protocol after xenotransplantation gave rise to four cell lines from among eight primary tumors and 5 from among 11 metastases, including four liver metastases. However, establishment of cell lines and xenografts was not significantly related to staging, metastasis location, or preoperative chemotherapy.

CT320/CT320X6 and CT329/CT329X12 cell line establishment. Among these 32 fragments, two tumor specimens, referred to as F320 and F329, were able to give rise to cell lines both directly and after xenografting, as illustrated in Fig. 1. The F320 fresh tumor gave rise to a stable cell line designated CT320 by direct *in vitro* establishment protocol and to an established xenograft referred to as XenoCT320. After successful serial passages, a fragment of XenoCT320 in turn led to a continuous cell line, the so-called CT320X6. Likewise, CT329 and CT329X112 cell lines were obtained from F329 and XenoCT329, respectively. CT320, CT320X6, CT329, and CT329X12 cell lines were grown in classic monolayers and were able to form full compact spheroids on agarose. Extensive characterization of these two couples of tumor cell lines was then done to compare matched low-passage human cell lines derived from a same tumor fragment by means of two different establishment protocols.

Morphologic studies of CT320 and CT320X6 cells. The morphologic characteristics of cell lines CT320 and CT320X6, as well as those of xenograft XenoCT320, were compared with characteristics of the primary cancer specimen (Fig. 1). Histologic examination of the primary cancer specimen and the xenograft revealed a moderately differentiated adenocarcinoma, with no major change between these two tissues. CT320 and CT320X6 showed multinucleated cells, an adherent growth pattern, and flattened polygonal morphology in accordance with their carcinoma origin. Neither grew strictly in monolayers: despite their epithelium-like morphology, after first forming polarized islets on plastic, they showed a tendency to pile up on top of the first cell layer. CT320 and CT320X6 spheroids showed a high degree of compaction and were resistant to mechanical disruption. Spheroid growth kinetics and morphology were identical to the spheroid features usually described (12). Sections of spheroids

clearly showed multicellular tumor spheroids, recapitulating the carcinoma phenotype close to the tumor architecture of their origin tissue.

***In vitro* growth characteristics of paired cell lines.** Growth properties of CT320 versus CT320X6 cell lines growing in monolayer cultures were compared. Cytofluorimetric analysis was carried out for cell cycle analysis. Propidium iodide staining showed no statistical differences between the two cell lines in terms of cell numbers in the G_0-G_1 (56.1 $\pm$ 4 versus 61 $\pm$ 6 for CT320 and CT320X6, respectively), S (26 $\pm$ 1 versus 25 $\pm$ 1), and G_2-M (14 $\pm$ 3 versus 10 $\pm$ 4) phases. In contrast, cell growth rates were significantly different after 7 days of culture (CT320: 2 $\pm$ 0.08 $\times$ 10^6 cells and CT320X6: 2.7 $\pm$ 0.23 $\times$ 10^6 cells, $P < 0.05$), showing that CT320X6 grew more rapidly than CT320. The Ki67 score, calculated from cell cultures immunostained against Ki67, was significantly higher in the CT320X6 cell line than in the CT320 cell line (13.7 $\pm$ 3% and 3.4 $\pm$ 2%, respectively, $P < 0.01$). Therefore, for a given time, identical proportions of CT320 and CT320X6 cells are in different cell cycle phases, but that these are shorter in the CT320X6 cell line. Similarly, cell growth rate of CT329X12 was statistically higher than the one of CT329 ($P < 0.05$).

Karyotype analysis of paired cell lines. Using classic cytogenetic techniques, a study of chromosomal abnormalities was done to investigate chromosomal similarities and/or differences between the cell lines derived from the same fragment but using two distinct protocols. In the four cell lines, intermetaphase variability existed, preventing the definition of each cell line by a single karyotypic formula. For example, chromosome number varied between 52 and 56 for CT320, between 49 and 55 for CT320X6, from 65 to 136 for CT329, and from 59 to 74 for CT329X12. Based on the most frequent chromosomal aberrations, the karyotypes are described in Fig. 2.

Complex karyotypes were indicated by the presence of both numerical and structural chromosomal aberrations. For each cell line, karyotypic evolution involved additional rearrangements and

Table 2. Clinicopathologic characteristics of the 32 human colon cancer specimens included in cell line establishment protocols

Tumor fragment	Preoperative chemotherapy	Tumor site	Primary tumor location	Primary tumor staging	Corresponding xenograft	Direct cell line establishment	Cell line establishment from xenograft
F305	No	Primary	Proximal	pT4N2M1	Positive	Negative	Positive
F306		Liver metastasis			Positive	Negative	Positive
F307		Ovarian metastasis			Positive	Negative	Positive
F308		Lymph node metastasis			Positive	Negative	Negative
F310	No	Liver metastasis 1	Proximal	pT4N2M1	Positive	Negative	Positive
F311		Liver metastasis 2			Negative	Negative	NT
F312	Yes	Primary	Distal	pT3N1M1	Negative	Negative	NT
F314	No	Primary	Proximal	pT3N1M1	Positive	Negative	Positive
F315		Liver metastasis			Negative	Negative	NT
F320	Yes	Primary	Distal	pT4N2M1	Positive	Positive	Positive
F316		Liver metastasis 1			Positive	Negative	Positive
F317		Liver metastasis 2			NT	Negative	NT
F318		Liver metastasis 3			NT	Negative	NT
F319		Carcinomatosis			Positive	Negative	Negative
F322	No	Primary	Distal	pT3N0M1	Positive	Negative	Negative
F323		Liver metastasis 1			Negative	Negative	NT
F327	No	Carcinomatosis	Proximal	pT4N2M1	NT	Negative	NT
F329	Yes	Primary	Distal	pT3N1M1	Positive	Positive	Positive
F330		Liver metastasis			Negative	Negative	NT
F335	Yes	Liver metastasis	Distal	pT3N1M1	NT	Negative	NT
F336	Yes	Primary	Proximal	pT3N0M1	Positive	Negative	Negative
F337		Liver metastasis			Positive	Negative	Negative
F338	Yes	Primary	Distal	pT4N2M1	Positive	Negative	Negative
F339		Liver metastasis			Positive	NT	Positive
F340	No	Primary	Proximal	pT4N2M1	Positive	Negative	NT
F341		Liver metastasis			Negative	Negative	NT
F342		Carcinomatosis			Positive	Negative	Negative
F343	Yes	Primary	Distal	pT3N1M1	Positive	Positive	Negative
F344		Liver metastasis			Positive	Negative	Negative
F345		Lymph node metastasis			Positive	Negative	Negative
F350	Yes	Primary	Proximal	pT3N1M1	NT	Negative	NT
F351		Liver metastasis			NT	Negative	NT
No. cell line or xenograft established/number of tested specimens					20/26	3/31	9/19

Abbreviation: NT, not treated.

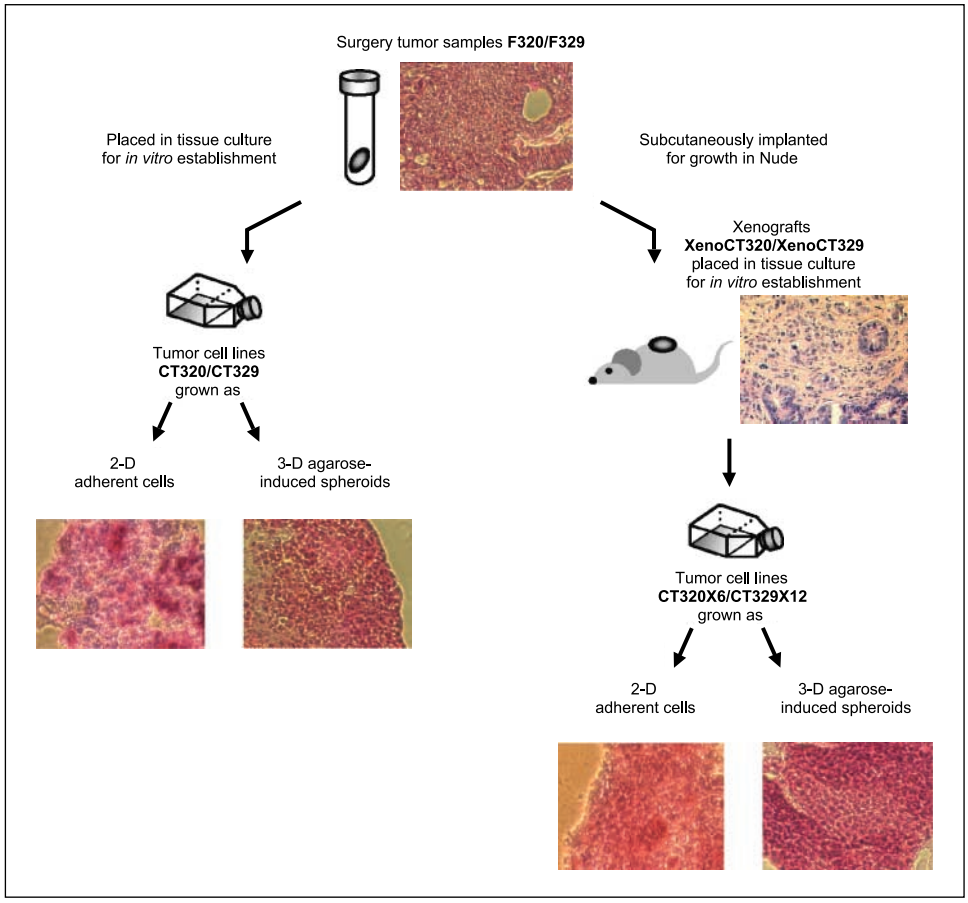

Figure 1. Experimental protocol leading to establishment of two pairs of cell lines, CT320/CT320X6 and CT329/CT329X12, from the colon cancer fragments F320 and F329 and the corresponding xenografts XenoCT320 and XenoCT329. Histologic and cytologic pictures of the primary cancer F320, its xenograft XenoCT320, and derived cell lines CT320 and CT320X6 in two-dimensional and three-dimensional architecture illustrated the steps of this protocol (H&E staining).

gains in chromosomes. These composite karyotypes confirmed that cell lines from F320 and F329 were not cross-contaminated but derived from two different tumors. They revealed also that chromosomal instability was more frequent for the cell lines obtained directly from patient's tumor than for their matched cell lines established from the xenografts. Moreover, karyotype analysis showed that CT320/CT320X6 and CT329/CT329X12, respectively, were paired but distinct cell lines derived from the same origin, as illustrated in Fig. 2.

***In vitro* sensitivity to chemotherapeutic agents.** To compare the sensitivities of paired cell lines to antitumor drugs, exponentially growing tumor cells displaying two-dimensional and three-dimensional architecture were treated for 3 days with 5-FU, CPT11, or L-OHP alone, and the IC_{50} levels were evaluated (Table 3). Tumor fragment F320 came from a patient who had unsuccessfully been given FOLFOX and FOLFIRI treatment; the combination of 5-FU, CPT11, and L-OHP thus led to no benefit for this patient. Tumor fragment F329 came from a patient who displayed a high response to FOLFOX treatment, but because of L-OHP–induced neurotoxicity, 5-FU was after given alone but unsuccessfully.

Whatever the *in vitro* model, CT320X6 cells were statistically more resistant to 5-FU and CPT11 than CT320, and CT329X12 cells were more sensitive to L-OHP than CT329 cells.

In addition, IC_{50}s of CT320 and CT320X6 cells in two-dimensional and three-dimensional models were compared for each drug to estimate the influence of cell architecture on drug sensitivity. Cell architecture had an effect on the sensitivity of CT320, CT320X6, and CT329 to 5-FU, on the sensitivity of CT320 and CT329X12 to L-OHP, and on the sensitivity of CT320X6 to CPT11. For the other experimental conditions, it was observed a high resistance to drugs, which did not allow discriminating between cell culture models.

Taken together, cell lines derived from F329 specimen were more resistant to the three drugs tested than cell lines derived from F320 specimen.

Gene expression profiling of cell lines and paired xenografts. Using real-time kinetic quantitative PCR, the expression profiles of 66 genes listed in Table 1 and known to be involved in the malignant process were studied in CT320 and CT320X6 displaying two-dimensional and three-dimensional architecture, XenoCT320, and F320. These genes were selected for their role in six essential alterations in cell physiology that collectively participate in oncogenesis as defined by Hanahan et al. (1, 16). Oligonucleotide primers and Taqman probes used were human specific and did not amplify cDNA of mouse origin. Each tissue or cell specimen was characterized by a gene expression pattern, defined as the set of normalized validated expressions of studied genes. Pearson correlation coefficients between gene expression patterns were calculated for comparison of gene expression among CT320, CT320X6, XenoCT320, and primary colon cancer F320 (Table 4). A similar approach using a set of 17 genes among the 66 genes (*BAD, BCL2, CCND1, CCNG2, CDC42, CDH1, CDKN1A, CDKN2B, ERBB2, FAS, KRAS, MMP9, MYC, PLAUR, TGFB1, VEGFC*, and *VIM*) was done to determine mRNA levels in the other cell lines established from xenografts and/or patient tumor, the corresponding xenografts, and tumor specimens (Table 4). These

17 genes have been chosen because gene expression patterns obtained with either 66 genes or 17 genes led to similar statistical results for F320 and its derived tissue and cells.

Whatever the *in vitro* culture model, gene expression patterns were correlated between CT320 and CT320X6. Nevertheless, better-fit correlations were observed for cell lines cultured in a given *in vitro* model: on one hand, between CT320 in two-dimensional and CT320X6 in two-dimensional (r = 0.951); and on the other hand, between CT320 in three-dimensional and CT320X6 in three-dimensional (r = 0.791). For gene expression pattern of patient tumor specimens, only three of seven patterns studied correlated with the matched xenograft and cell lines (F305, F310, and F314). The gene expression pattern of the xenografts was consistently correlated with that of the corresponding cell lines established from the xenograft but was not correlated with that of the corresponding cell lines directly established from the patient tumor fragment.

Discussion

Well-characterized low-passage colon cancer cell lines are rare, and this paucity of relevant biological materials hampers the investigation of colorectal carcinogenesis and the testing of new therapeutic strategies. In this context, the first goal of the present study was to elaborate an experimental strategy to improve the success of cell line establishment. In experiments done on 32 surgical colon cancer specimens, it was observed that the establishment of cell lines from tumor xenografts after a small

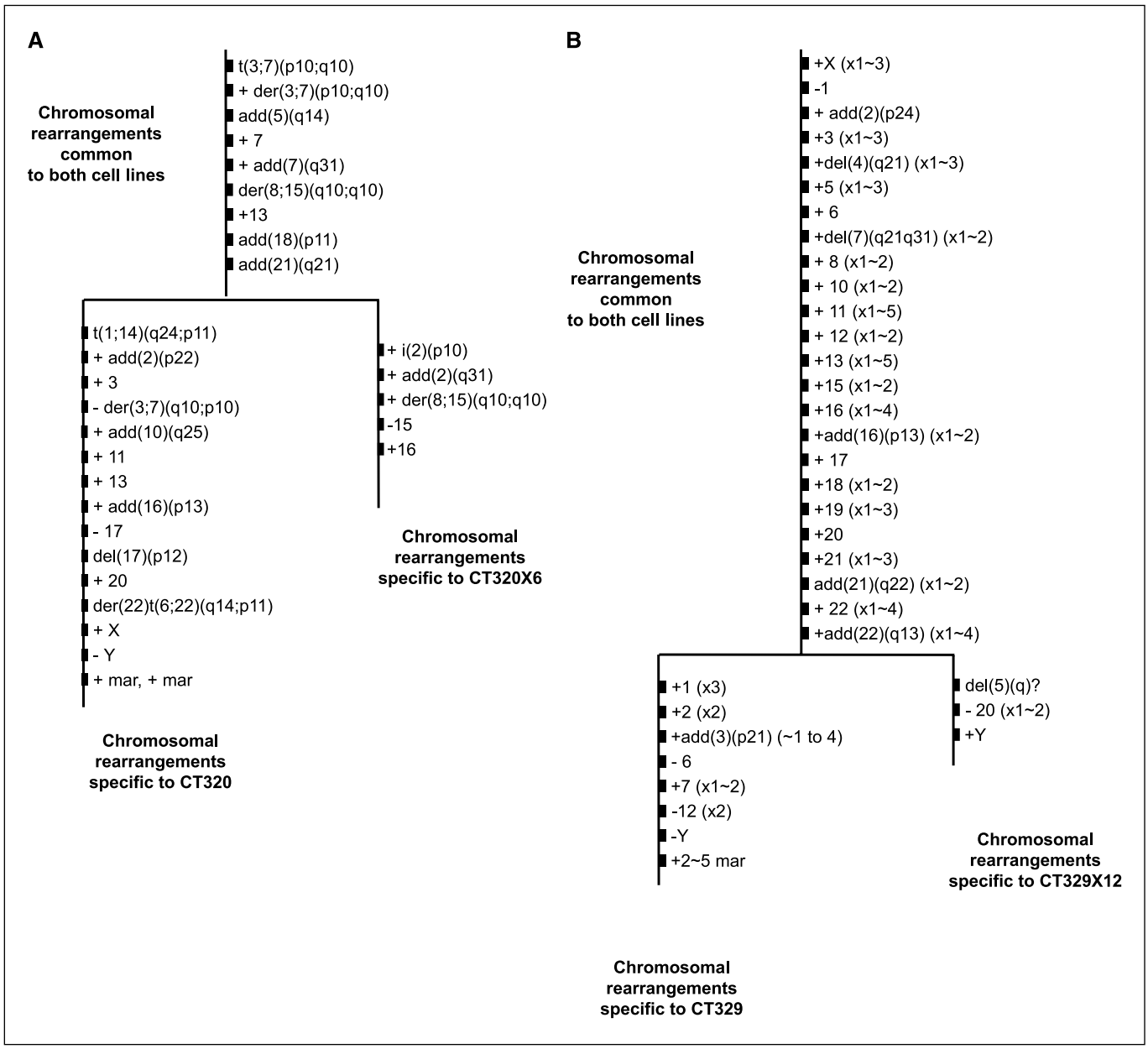

Figure 2. Cytogenetic features of the two pairs of cell lines, CT320/CT320X6 and CT329/CT329X12. Composite chromosomal karyotypes showed rearrangements common to both cell lines and rearrangements specific to each cell line.

Table 3. IC_{50}s for 5-FU, CPT11, and L-OHP

Cell lines	IC_{50} (μmol/L)								
	5FU			CPT11			L-OHP		
	2D	3D	*P*	2D	3D	*P*	2D	3D	*P*
CT320	48 ± 8	28 ± 3	<0.05	19 ± 2	10 ± 2	<0.01	27 ± 3	8 ± 1	<0.05
CT320X6	120 ± 18	>200	<0.01	58 ± 7	>200		35 ± 2	38 ± 7	
P	<0.05	<0.01		<0.01	<0.01		—	<0.01	
CT329	>200	44 ± 10	<0.01	>200	>200		>200	>200	<0.01
CT329X12	>200	>200	—	>200	>200		86 ± 3	51 ± 5	
P	—	<0.01		—	—		<0.01	<0.01	

NOTE: Data were reported as means ± SE (at least three independent experiments). Significant differences between treated groups were evaluated using the unpaired Student's *t* test.
Abbreviations: 2D, two-dimensional; 3D, three-dimensional.

number of passages was more successful than direct *in vitro* establishment from clinical specimens: 9 cell lines were established from 19 xenografts, whereas only 3 cell lines were generated from 31 fresh surgically removed tumors. In addition, serially transplantable xenografts were frequently established: 20 of 26 fragments grafted in mice led to established xenografts. Human colon carcinoma grafting into immunodeficient mice to obtain serially transplantable xenografts has frequently been reported but rarely used for secondary *in vitro* cell line establishment (17–19). The rate of xenograft and/or subsequent cell line success reported here seems to be as high as those reported by other investigators. Likewise, numerous studies have reported the difficulty to initiate cancer cell lines from fresh surgically removed colon tumors (20–23), and our 9.7% success rate compares favorably with other attempts to establish cell lines from a large number of colorectal adenocarcinomas (22, 24). However, any study has reported neither both xenografting and direct *in vitro* cell line establishment from the same surgical specimens nor characterization of cell lines obtained by the two processes. Consequently, combination of the xenograft step with conventional cell line protocol using fresh human colon tumors was here shown for the first time to lead to the obtaining of a greater number of tumor cell lines from a larger range of tumor samples than has been previously reported. Thus, a large bank of colon cancer cell lines from a wide panel of human colon cancer specimens may reflect the diversity of tumor phenotypes and could provide adequate models for studying colon cancer disease heterogeneity (6). Moreover, we showed that the xenograft step was necessary to more efficiently obtain colon cancer cell lines from metastatic sites: 5 cell lines were obtained from among 11 xenografted metastases, whereas none of 20 metastases gave rise to cell lines by direct *in vitro* establishment protocol. This suggests that tumor fragments from metastasis are more difficult to culture *in vitro*. It is noteworthy that 21 of the 29 human colon cancer cell lines from the American Type Culture Collection (Rockville, MD) came from primary tumors, whereas only five originated from metastases and none from liver metastases (the three remaining cell lines represented substrains from preceding cell lines): tumor fragments from metastasis would be more difficult to culture *in vitro*. Similarly, colorectal cancer cell lines established by other authors more often originated from primary tumors or sites of metastasis, such as ascites, the abdominal wall, or the lymph nodes (20–23). Only a few were derived from liver metastases (18, 23), although this location corresponds to a frequent colon carcinoma dissemination site.

The present experiments also provided a unique opportunity for performing a comparative study of two pairs of cell lines generated from the same surgical specimen and established directly *in vitro* or through a xenograft. Indeed, among the 29 human colon cancer cell lines from the American Type Culture Collection, only a pair of those cell lines (CCL-221 and CCL-225) may have been generated from the same cancer specimen (25). The present comparative study was carried out on two surgical colon cancer fragments, which led to continuous cell lines CT320 and CT329 and to serially transplantable tumors XenoCT320 and XenoCT329, which in turn gave rise to the permanent cell lines CT320X6 and CT329X12. CT320 versus CT320X6 cells and CT329 versus CT329X12 were compared in terms of morphology, growth, karyotype, gene expression profiles, and chemosensitivity to anticancer drugs. Experiments were also conducted to compare the biological features of cells cultured in two-dimensional and three-dimensional conformations. Use of cancer cells in three-dimensional cell cultures is considered as a biologically relevant model to investigate cancer initiation and progression (26, 27). No morphologic change was observed in a cytologic study, although colon cancer cell lines may show widely varying morphologies (28). Likewise, global gene expression profiles did not significantly differ between these cell lines. The global gene expression profile was based on a quantification of genes selected for their role in essential alterations that collectively dictate the malignant process (1). The global statistical analysis showed that no dramatic change in gene expression was introduced by the two protocols of cell line establishment. This gene expression analysis also showed that gene profiles of pairs of xenograft/cell line established from the xenograft were always correlated.

Differences between the paired cell lines were seen in karyotype, growth kinetics, and chemosensitivity. Karyotypes showed a pattern of aberrations indicating that these cell lines share a core of karyotype alterations and display distinctive additional chromosomal aberrations because no chromosomal change can be regarded as a common primary event in colon cancer carcinogenesis

Table 4. Correlation coefficients (*r*) for comparison of gene expression in cell lines and matched xenograft and patient tumor fragments (Pearson correlation)

	CT320				CT320X6				XenoCT320		F320	
	2D		3D		2D		3D					
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
CT320												
2D	—	—	0.402	<0.01	0.952	<0.01	0.433	<0.01	0.215	>0.1	0.041	>0.1
3D	0.402	<0.01	—	—	0.451	<0.01	0.790	<0.01	0.459	<0.01	0.105	>0.1
CT320X6												
2D	0.951	<0.01	0.451	<0.01	—	—	0.551	<0.01	0.485	<0.01	0.008	>0.1
3D	0.433	<0.01	0.790	<0.01	0.551	<0.01	—	—	0.416	<0.01	0.058	>0.1
XenoCT320	0.215	>0.1	0.459	<0.01	0.485	<0.01	0.416	<0.01	—	—	0.587	<0.01
F320	0.041	>0.1	0.105	>0.1	0.008	>0.1	0.058	>0.1	0.587	<0.01	—	—

	CT329				CT329X12				XenoCT329		F329	
	2D		3D		2D		3D					
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
CT329												
2D	—	—	ND	ND	0.846	<0.01	0.726	<0.01	0.299	>0.1	0.247	>0.1
3D	ND	ND	—	—	ND	ND	ND	ND	ND	ND	ND	ND
CT329X12												
2D	0.846	<0.01	ND	ND	—	—	0.813	<0.01	0.523	<0.05	0.046	>0.1
3D	0.726	<0.01	ND	ND	0.813	<0.01	—	—	0.789	<0.01	0.148	>0.1
XenoCT329	0.299	>0.1	ND	ND	0.523	<0.05	0,789	<0.01	—	—	0.308	>0.1
F329	0.247	>0.1	ND	ND	0.046	>0.1	0.148	>0.1	0.308	>0.1	—	—

	CT316X5				XenoCT316		F316	
	2D		3D					
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
CT316X5								
2D	—	—	0.963	<0.01	0.945	<0.01	0.902	<0.01
3D	0.963	<0.01	—	—	0.901	<0.01	0.242	>0.1
XenoCT316	0.945	<0.01	0.901	<0.01	—	—	0.301	>0.1
F316	0.247	>0.1	0.242	>0.1	0.301	>0.1	—	—

	CT305X1		XenoCT305		F305	
	r	*p*	*r*	*p*	*r*	*p*
CT305X1	—	—	0.947	<0.01	0.863	<0.01
XenoCT305	0.947	<0.01	—	—	0.912	<0.01
F305	0.863	<0.01	0.912	<0.01	—	—

	CT306X1		XenoCT306		F306	
	r	*p*	*r*	*p*	*r*	*p*
CT306X1	—	—	0.936	<0.01	ND	ND
XenoCT306	0.936	<0.01	—	—	ND	ND
F306	ND	ND	ND	ND	—	—

(*Continued on the following page*)

Table 4. Correlation coefficients (*r*) for comparison of gene expression in cell lines and matched xenograft and patient tumor fragments (Pearson correlation) (Cont'd)

	CT307X1		XenoCT307		F307	
	r	*p*	*r*	*p*	*r*	*p*
CT307X1	—	—	0.818	<0.01	ND	ND
XenoCT307	0.818	<0.01	—	—	ND	ND
F307	ND	ND	ND	ND	—	—
	CT310X6		XenoCT310		F310	
	r	*p*	*r*	*p*	*r*	*p*
CT310X6	—	—	0.860	<0.01	0.848	<0.01
XenoCT310	0.860	<0.01	—	—	0.815	<0.01
F310	0.848	<0.01	0.815	<0.01	—	—
	CT314X6		XenoCT314		F314	
	r	*p*	*r*	*p*	*r*	*p*
CT314X6	—	—	0.936	<0.01	0.866	<0.01
XenoCT314	0.936	<0.01	—	—	0.519	<0.01
F314	0.866	<0.01	0.519	<0.05	—	—
	CT339X		XenoCT339		F339	
	r	*p*	*r*	*p*	*r*	*p*
CT339X	—	—	0.973	<0.01	0.400	>0.1
XenoCT339	0.973	<0.01	—	—	0.430	>0.1
F339	0.400	>0.1	0.430	>0.1	—	—

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; ND, not done.

(29). The commonality of these chromosomal aberrations cell lines is due to their common origin and not to specific features of colon cancer. It is noteworthy that CT320, CT320X6, CT329, and CT329X12 cells were studied at early *in vitro* passages (up to 10–15), reflecting the initial alterations in this tumor and not a genotypic diversity, which could have been attributed to long-term *in vitro* cell culture (30). Chemosensitivity tests underscored the fact that CT320 versus CT320X6 and CT329 versus CT329X12 were distinct cell lines. Three drugs, representing reference adjuvant therapies in colon cancer, were tested (31). CT320X6 showed higher resistance than CT320 to anticancer drugs whatever the *in vitro* model, except when the CT320X6 monolayer was treated with L-OHP. CT329X12 was also more resistant to 5-FU in three-dimensional conformation than CT329 but more sensitive to L-OHP whatever the *in vitro* model. Spheroids were used in chemosensitivity tests because three-dimensional spheroids were primarily used to study tumor resistance to radiotherapy and chemotherapy (32). More recently, Weaver et al. showed that polarized three-dimensional architecture confers upon the tumor cell resistance to apoptosis-inducing agents, including chemotherapy drugs and ligation of cell death receptors (33). The most striking differences in chemosensitivity tests between the cell lines were observed in this three-dimensional culture model.

These results also point to an heterogeneity at the level of a unique tumor site because surgical tumor samples F320 and F329 gave rise to two pairs of distinct but closely related cell lines. Variability in genotype and phenotype of low-passage colon cancer cell lines, reflecting the diversity of the parental colon cancer cells *in vivo*, has already been reported (21–23, 28). However, the diversity described in the latter studies was generally related to specimens originating from colon carcinomas collected from several patients and/or from specimens collected at different locations, whereas the present study further highlights biological intratumoral heterogeneity observed at a unique site. Previous observations of diverse cell populations in individual colon cancers were also supported by the isolation of sublines with distinct karyotypic and growth characteristics (34). In the present study, the differences in the two *in vitro* cell line establishment protocols may have favored distinct tumor cell populations.

Finally, xenotransplantation of patient tumor fragments into immunocompromised mice before *in vitro* establishment protocol increases the success rate in generating human cancer cell lines from both primary colon cancers and liver metastases. The availability of a large series of novel cell lines for human tumors, mimicking biological heterogeneity (observed in colon cancer and intrinsically in patient tumors), would enable both detailed

fundamental investigation of colon cancer cell biology and experimental exploration of treatment approaches.

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