Original Study

E-Cadherin in Colorectal Cancer: Relation to Chemosensitivity

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Abstract

We analyzed colorectal cancer chemosensitivity according to expression of the main epithelial–mesenchymal transition (EMT) marker, E-cadherin. Human colon adenocarcinoma cell lines HT29 and HCT116 and 14 primary short-term cultures from patient tumors were used. Increased chemosensitivity of the cell line with EMT phenotype, HCT116, was demonstrated; this may serve as a predictive marker of chemotherapy efficacy.

Background: The conventional chemotherapy of colorectal cancer with irinotecan, 5-fluorouracil, and oxaliplatin remains one of the front-line treatments worldwide. However, its efficacy is quite low. Recently studies of the epithelial–mesenchymal transition (EMT) have become the focus of investigations into the cause of chemoresistance in several types of cancer, including colorectal cancer. The data about the role of EMT in chemosensitivity are controversial. **Materials and Methods:** Human colon adenocarcinoma cell lines HT29 and HCT116 and 14 primary short-term cultures established from patient tumors were used. The chemosensitivity to irinotecan, 5-fluorouracil, and oxaliplatin was assessed using the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Immunocytochemistry, immunohistochemistry, and Western blot test were used to investigate the E-cadherin expression, the loss of which is a major hallmark of EMT. **Results:** Elevated chemosensitivity of the cell line with EMT phenotype, HCT116, was demonstrated. Increased chemosensitivity was revealed in HT29 cell line upon EMT induction. E-cadherin–positive short-term cultures were more resistant to all the drugs tested, whereas each of E-cadherin–negative cultures showed sensitivity to at least one drug. The statistically significant dependency of cells viability on the E-cadherin expression ($P < .04$) was demonstrated on the short-term cultures using 2 concentrations of each drug. **Conclusion:** The data obtained may serve as a basis for the analysis of colon cancer chemosensitivity using short-term cultures and the assay of E-cadherin expression.

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed types of cancer in the world, often presenting with advanced and metastatic disease.[1,2] Despite advances in chemotherapeutic agents and the addition of targeted therapies to such chemotherapy, the prognoses for patients with metastatic CRC have only slightly improved.[3-7] Intrinsic (or de novo) and acquired forms of chemotherapy resistance are now considered to be the major challenges limiting therapeutic success.[2,4,8] A clear understanding of such innate resistance is essential for effective treatment.[2] This has been demonstrated when epidermal growth factor receptor antagonists were used in additional studies to reveal the primary cause of chemoresistance, thereby enabling a vast improvement to be achieved in the clinical outcome.[9]

Recently studies of the epithelial–mesenchymal transition (EMT)—the process of transformation of epithelial cells into spindle fibroblast-like cells with the gain of mesenchymal features—have become the focus of investigations into the cause of chemoresistance in several types of cancer.[8,10-12] It is well established that EMT enhances proliferation, mobility, and the invasion of epithelial cells that results in the appearance of distant metastases.[11,13,14] The relationships between the EMT and chemosensitivity are not fully understood. The high sensitivity of rapidly proliferating cells to chemotherapeutic drugs, especially to cytotoxic drugs, is well known.[15,16] Yet only a few studies have shown an increase in the chemosensitivity of cancer cells with features of EMT—for example, in prostate, breast, and colorectal

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cancers.[17,18] Nevertheless, a number of studies have demonstrated a decrease in chemosensitivity for different cancer types, including CRC, during EMT.[6,10,19-21] Thus, it could be concluded that the findings are contradictory and do not provide a clear answer about the role of EMT in chemosensitivity, particularly in CRC.

The main marker of EMT is the loss of membrane E-cadherin expression.[11,14] E-cadherin is a Ca-dependent glycoprotein required for efficient intercellular adhesion. In addition, it acts as a suppressor gene for tumor growth and tumor progression.[6,18] Previously, several studies had demonstrated that E-cadherin acts as a chemoprotective agent by blocking drug diffusion through the intercellular junctions.[18,22,23] This, however, contradicts the decreased chemosensitivity in cells evidencing EMT. So the role of the chemosensitivity of EMT in association with the expression of E-cadherin remains of great interest.

The aim of our study was to clarify the implication of EMT accompanied by E-cadherin expression in the chemosensitivity of CRCs. The study was performed using low-passage human cancer cell lines established from patient tumors. Although numerous studies have suggested that immortal cancer cell lines only poorly represent the diverse, heterogeneous, and drug-resistant tumors occurring in patients, short-term cultures of primary cells have gained importance in personalized cancer therapy.[24,25] The chemosensitivity to 3 drugs primarily used for the chemotherapy of CRC—5-fluorouracil (5-FU), oxaliplatin, and irinotecan—was investigated. First, the relation between chemosensitivity and E-cadherin and vimentin expression was established for 2 human CRC cell lines, HT29 (E-cadherin positive) and HCT116 (E-cadherin negative). Then EMT was induced in the HT29 cell line, resulting in an increase in chemosensitivity. To assess the chemosensitivity of the short-term cancer-cell cultures isolated from patient tumors, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed using drug concentration causing 50% inhibition (IC_{50}) values determined for HCT116 and HT29. The correlation of cell viability with the expression of E-cadherin for 14 short-term cell cultures was assessed. The E-cadherin expression was additionally verified by immunohistochemical (IHC) assay of the original tumors.

To our knowledge, this is the first assessment of the correlation between E-cadherin expression and the chemosensitivity of CRC cells isolated from patients.

Materials and Methods

Cell Cultures

Human colon adenocarcinoma cell lines HT29 and HCT116 were routinely grown in Eagle/Dulbecco modified Eagle medium (DMEM) (PanEko, Russia) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), 2 mM glutamine, 10 mg/mL penicillin, and 10 mg/mL streptomycin, while the HCT116 cell line was cultured in DMEM (Gibco, Life Technologies, USA) supplemented with 10% FBS (HyClone), 2 mM glutamine, 10 mg/mL penicillin, and 10 mg/mL streptomycin at 37°C in a 5% CO_2 humidified atmosphere.

Patient Tumors

Colon cancer samples were obtained from the Volga Regional Medical Center in accordance with protocols approved by the local ethical committee. All the patients selected for study had histologically demonstrated CRC and had not been pretreated with radiotherapy or chemotherapy. The surgical specimens were sufficiently large to be able to initiate short-term tumor cultures and to provide a specimen for paraffin embedding ready for IHC analysis. Tumor material was delivered to the laboratory, on ice, in a medium containing elevated levels of antimicrobial agents (DMEM/F-12 supplemented with 20 mg/mL penicillin, 20 mg/mL streptomycin, 20 mg/mL amphotericin, and 20 mg/mL gentamicin).

Establishment of Human Primary Colon Cancer Cell Lines

Tumor samples were processed using standard protocols for tumor cultures in vitro,[26,27] adapted for the isolation of primary cell cultures from colon tumors.

The colon tumor samples were washed 7 to 10 times in wash medium (DMEM/F-12 supplemented with 20 mg/mL penicillin, 20 mg/mL streptomycin, 20 mg/mL amphotericin, 20 mg/mL gentamicin) and in Hanks solution without Ca^{2+} or Mg^{2+} containing the same antibiotics. Then the tumor fragments were placed in a 70 mm petri dish in a small volume of wash medium, and any necrotic areas, fat, blood vessels, cartilage, and surrounding normal tissue were removed. The tumor tissue was then chopped with blades into small pieces (1-2 mm) and placed into a sterile tube. Next, enzymatic digestion was performed using a mixture of Liberase (Roche, USA) and Hyaluronidase (Sigma, USA) in DMEM/F-12 for 45 minutes at 37°C. The mixture was resuspended every 10 to 15 minutes. The biomass was then filtered through a nylon mesh (100 μm), and an excess volume of fresh medium was added to neutralize the enzymes before the suspension was centrifuged at 1.6×10^3 rpm for 10 minutes. The supernatant was removed, and the cell pellet was resuspended in ACK lysing buffer (Gibco) according to the manufacturer's directions to remove red blood cells. Then the cells were extensively washed, resuspended, and centrifuged in fresh wash medium.

The tumor cells were cultured in 25 cm^2 or 75 cm^2 low-attachment culture flasks in DMEM/F-12 (Gibco) containing 1 μL/mL trace element B (Corning, USA), 1 μL/mL trace element C (Corning), 10 μL/mL HEPES (Gibco), 10 μL/mL insulin—transferrin—selenite (Gibco), 10 μL/mL sodium pyruvate (STEMCELL Technologies, Canada), 0.14 μL/mL heparin—sodium (Braun, Germany), 2 μL/mL lipid mixture 1 (Sigma), 10 μL/mL N2-supplement (Gibco), 20 μL/mL B27 supplement (Gibco), 0.2 μL/mL epidermal growth factor (PAN-Biotech, Russia), and 1 μL/mL basic fibroblast growth factor (STEMCELL Technologies) at 37°C, 5% CO_2. The cells were routinely passed once a week, and the medium was changed twice in between.

To simplify the work and increase the accuracy of the quantitative examination during the chemosensitivity assessment of the short-term cultures, the cells were transferred to a 2-D culture on plates precoated with collagen. For this purpose, collagen I (Gibco) was added to a 96-well plate (50 μL per well) a day before cell seeding.

Cell Viability Assay

To determine the cell viability, an MTT assay was performed. A total of 3×10^3 cells in 100 mL of culture medium was seeded onto 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were allowed to attach overnight; then the appropriate drug was added for 72 hours. The MTT assay

was performed in accordance with the manufacturer's directions, with the optical density being measured using a multimode microplate reader (Synergy Mx; BioTek Instruments, USA). The proportion of viable cells was calculated as the percentage of purple formazan-stained treated cells to that of the untreated control cells. The following drugs were used for the treatment: oxaliplatin (Oncotec Pharma Produktion, Germany), 5-FU (Lence Pharma, Russia), and irinotecan (Lemery, Mexico). For both the HT29 and HCT116 cell lines, MTT assays were performed with drug concentrations of 200 μM, 100 μM, 50 μM, 25 μM, 10 μM, 1 μM, and 0.1 μM, and the IC_{50} values were determined.

For the short-term cultures from patient tumors, the MTT assay was performed using 2 doses for each drug (IC_{50} values calculated for the HCT116 and HT29 cell lines). Although a determination of the IC_{50} values for the short-term cell cultures would be more informative in terms of chemosensitivity assessment, the quantity of isolated cells was insufficient to do this.

To avoid culture crisis, the chemosensitivity assay was performed on the short-term cultures of 2 to 3 passages, within 10 to 30 days after isolation of cells from tumor samples. Before chemosensitivity testing, a small amount of cell suspension was sampled and stained with trypan blue. The chemosensitivity was assessed if the percentage of live cells in the short-term culture was $> 70\%$.

For all cell cultures and drug doses, MTT tests were performed in 3 independent repetitions, each containing 7 to 10 replicate wells.

Induction of EMT

StemXVivo EMT Inducing Media Supplement (Bio-Techne, USA) was used for the induction of EMT according to the manufacturer's directions. HT29 (3000 cells per well) were seeded into 96-well plates in DMEM containing 10% FBS, antibiotics, and single-strength StemXVivo EMT Inducing Media Supplement. The cells were incubated at 37°C with 5% CO_2 for 5 days. The medium was changed 3 days after plating. The original cell line, cultured under standard conditions but without the addition of the EMT-inducing complement, was used as a control. Both cell cultures had the same number of passages.

Wound-Healing Assay

The method was adopted from Zhang et al[28] with some alterations. Cells were seeded in 6-well plates and cultured to 80% to 90% confluence. Subsequently, artificial wounds were created on the confluent cell monolayer using 200 μL pipette tips, and the detached cells were washed away twice with FBS-free culture medium. Then the cells were grown in FBS-free Eagle medium, and migrating cells were imaged under an inverted microscope at different time points (0, 72, 96, and 120 hours).

Immunocytochemistry

For immunocytochemical (ICC) staining, the cells were grown in 96-well plates for 24 hours after seeding and fixed in 4% formaldehyde for 15 minutes. The following primary antibodies were used: rabbit antibodies against E-cadherin (ab15148, Abcam, USA), mouse antibody Ki-67 (ab15580 Abcam), and mouse antibody anti-EpCam (ab20160, Abcam). Subsequently, cells were stained with fluorescein isothiocyanate (FITC)-labeled chicken anti-rabbit immunoglobulin (Ig) G secondary antibody (ab6825, Abcam) or Alexa Fluor 594—labeled goat anti-mouse IgG secondary antibody (ab6825, Abcam). Staining was performed according to the manufacturer's directions. In addition, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the cell nuclei. Fluorescence was observed using a Leica DM IL (Leica, Germany) fluorescence microscope equipped with the following filters: A4 UV BP 360/40 400 BP 470/40 for DAPI, CFP ET YFP ET (Ex: BP 500/20, Em: BP 535/30) for FITC and TX2 green BP 560/40 595 BP 645/75 for Alexa Fluor 594 labeling.

Immunohistochemistry

Tissue samples were placed in 10% formalin immediately after surgical resection for no longer than 48 hours, then embedded in paraffin. Sections (4 μm) of the paraffin blocks were prepared on a Leica RM2245 microtome. Primary antibodies to E-cadherin (ab15148, Abcam) and ImmPRESS HRP anti-rabbit IgG (Peroxidase) Polymer Detection Kit (Vector Laboratories, USA) were used according to the manufacturer's directions to obtain specific staining. All slices were counterstained with Mayers hematoxylin. Sections were rinsed 3 times with phosphate-buffered saline after each step of the procedure.[29]

Membrane-specific E-cadherin staining was assessed using a Leica DM 2500 microscope. To compare the E-cadherin stained to the control (nonstained) samples, the immunoreactivity in 1000 tumor cells in a total of 10 fields of view was evaluated for each specimen at 400× magnification. A threshold of $> 10\%$ was taken to consider cells E-cadherin positive. The specimens were assigned scores for immunoreactivity ranging from 0 to 4, where 0 is $\leq 10\%$, 1 is 11-30%, 2 is 31-60%, 3 is 60-80%, and 4 is $> 80\%$ stained cells.[13]

Western Blot Analysis

Cells were lysed on ice in radioimmunoprecipitation assay buffer (pH 7.4) completed with protease inhibitor cocktail (Roche Diagnostics, USA). Then proteins were precipitated with ice-cold acetone and stored until analysis. Before immunoblotting, samples were heated in Laemmli sample buffer at 95°C for 5 minutes.

Protein samples (20 μL per well) and molecular weight ladder (26619, Thermo Fisher Scientific, USA) were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel for approximately 45 minutes at 20 mA in running buffer (0.125 M Tris base, 0.96 M glycine, 0.5% SDS, pH 8.3). The immunoblotting was performed using polyvinyl difluoride Immobilon-P membrane (Merck Millipore, UK), the Mini Trans-Blot cell system (Bio-Rad, USA) and SNAP i.d. 2.0 Protein Detection System (Merck Millipore). The primary antibodies to E-cadherin (ab15148, Abcam) or vimentin (ab16700, Abcam) with subsequent peroxidase-conjugated secondary antibodies (anti-rabbit IgG; Sigma) were used. After each incubation, the excess of primary/secondary antibodies was removed by washing the membranes in phosphate-buffered saline—Tween 20 four times. Then the membranes were exposed to Clarity enhanced chemiluminescence (ECL) reagent (GE Healthcare, USA). Signals were detected in photographic film (Amersham Hyperfilm ECL; Amersham, USA) and estimated by a GelAnalyser (Istvan Lazar, Hungary).

Statistical Analysis

All data are expressed as mean values ± standard deviation. The Student *t* test was used to analyze statistically significant differences

between values, with $P \leq .05$ being considered as significant for standard cancer cell lines. The dependence of chemosensitivity on E-cadherin expression in short-term primary cell cultures was assessed using the nonparametric Kolmogorov-Smirnov test for small samples.

Results

Chemosensitivity and E-Cadherin/Vimentin in HCT116 and HT29 Cell Lines

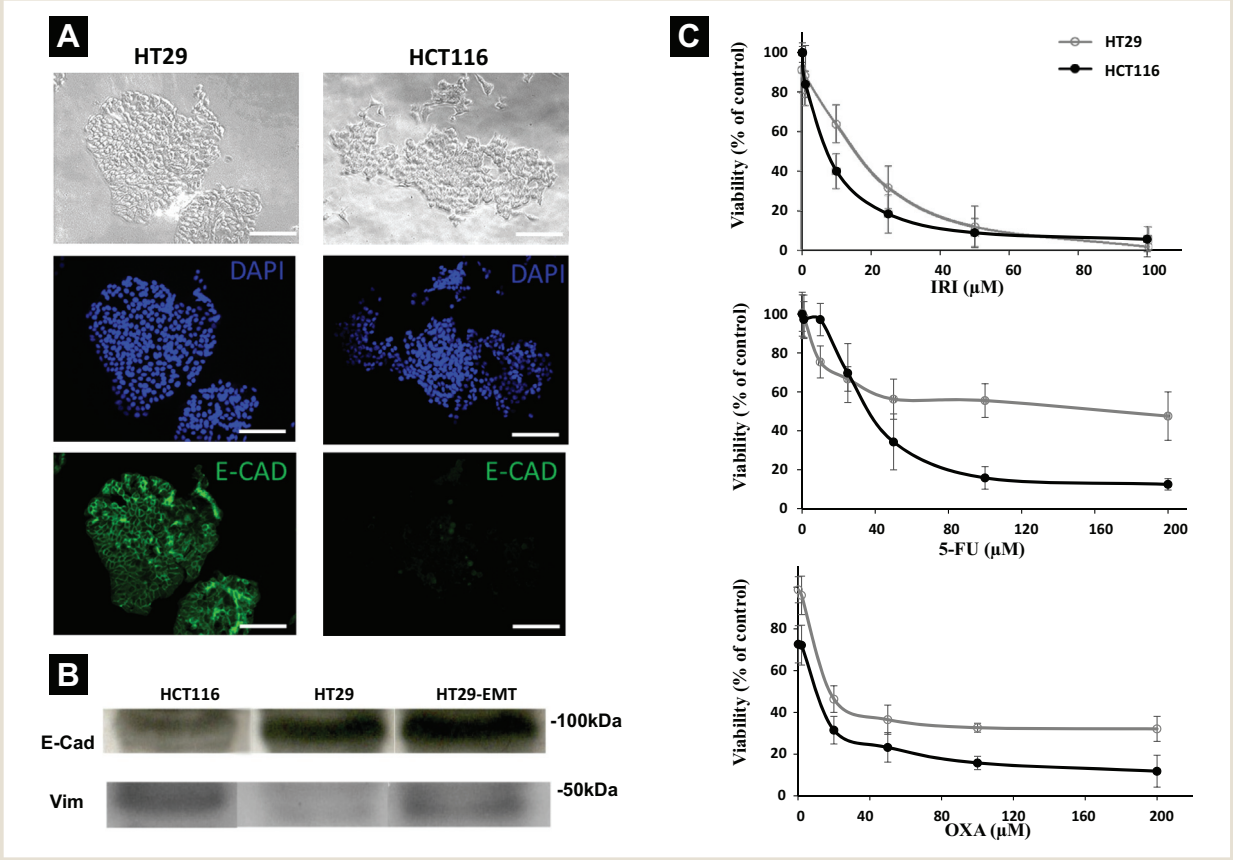
We first tested the chemosensitivity of 2 colon cancer cell lines, HCT116 and HT29, which are markedly different in morphology, physiology, and expression of EMT markers. HCT116 cells are fast proliferating and loosely adherent, with spindlelike morphology and low E-cadherin expression (Figure 1A, B). Cells in the HT29 line poses much a slower proliferation rate, form compact colonies of densely packed cells, and have marked E-cadherin expression in the plasma membrane (Figure 1A, B). Moreover, HT29 cells show down-regulation of the mesenchymal marker vimentin. Therefore, morphologic ICC analysis and immunoblotting confirmed that HT29 cells did not undergo EMT, in contrast to HCT116 cells.

The chemosensitivity of these cultures to oxaliplatin, 5-FU, and irinotecan was analyzed by MTT assay. For HT29, the IC_{50} values were 4.7 ± 0.7 μM for oxaliplatin, 190.0 ± 25.5 μM for 5-FU, and 22.5 ± 3.5 μM for irinotecan. By comparison, HCT116 cells had IC_{50} values of 2.4 ± 0.5 μM for oxaliplatin, 19.9 ± 2.3 μM for 5-FU, and 3.2 ± 0.7 μM for irinotecan (Figure 1A). Thus, the HT29 cell line demonstrated clearly reduced sensitivities to all 3 chemotherapeutic drugs. Therefore, the elevated chemosensitivity of cancer cells with the EMT phenotype was demonstrated using 2 standard colon cancer cell lines.

Chemosensitivity After Inducing EMT in HT29 Cell Line

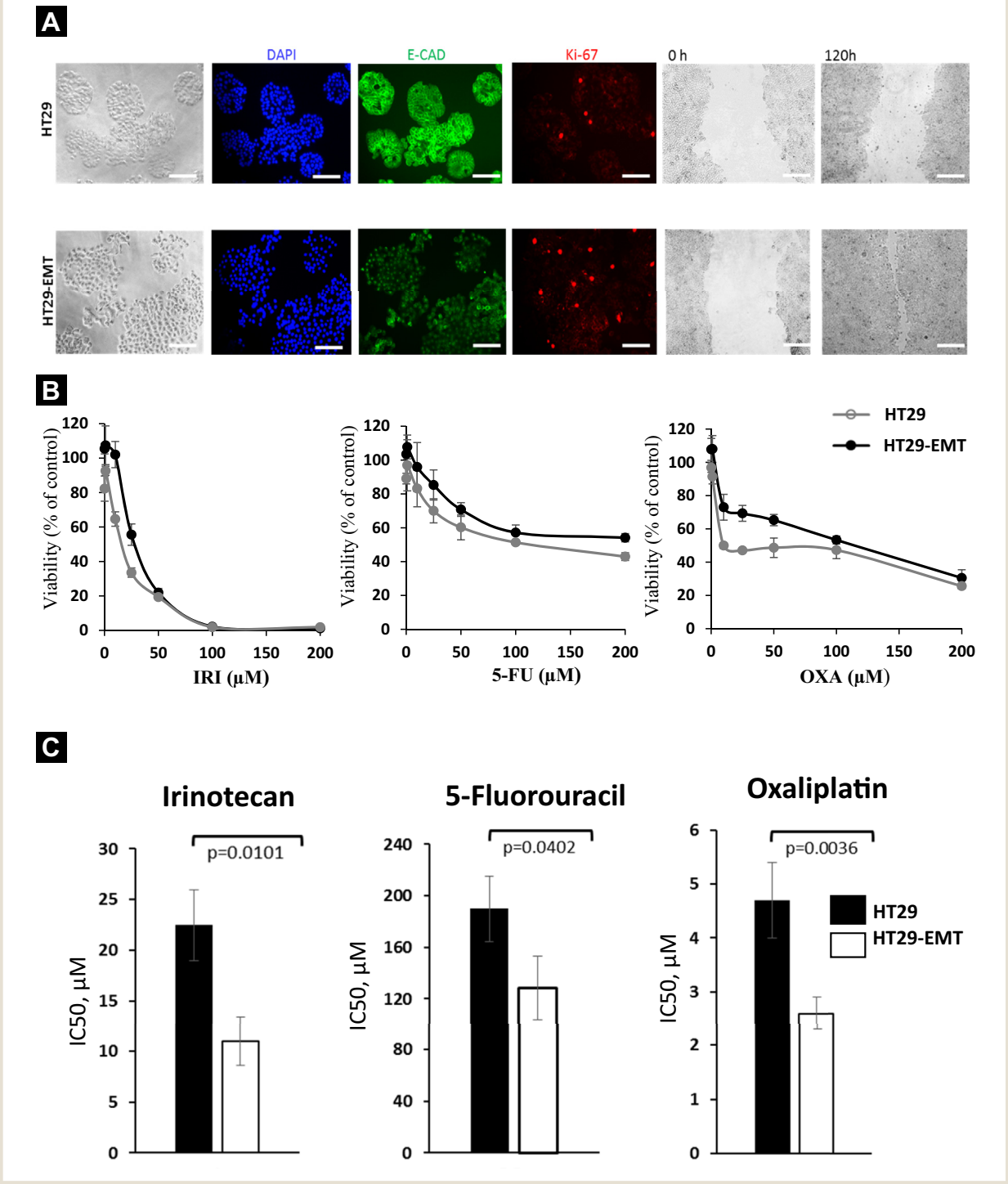
To be convinced of the link between the chemosensitivity and E-cadherin expression, EMT was induced in the HT29 cells using EMT-inducing media supplement. The development of EMT was confirmed by morphologic, ICC, and functional analysis. The

Figure 1 Analysis of HT29 and HCT116 cell lines. (A) phase-contrast microphotographs and immunocystochemical analyses of HT29 and HCT116 cells. (B) Western Blotting of E-Cadherin and Vimentin in HCT116 and HT29 Cell Lines, and HT29 Cell Line After EMT Induction. (C) Cell Viability in Presence of IRI, 5-FU, or OXA for HT29 and HCT116 Cell Lines. Green (FITC) Indicates E-Cadherin Expression; Blue (DAPI), Nuclei Staining. Scale bar, 100 μm



Abbreviations: DAPI = 4′,6-diamidino-2-phenylindole; EMT = epithelial–mesenchymal transition; 5-FU = 5-fluorouracil; FITC = fluorescein isothiocyanate; IRI = irinotecan; OXA = oxaliplatin.

Figure 2 Induction of EMT in HT29 Cells. (A) Morphologic and Immunocytochemical Analysis of Control HT29 Cells and HT29 Cells With Induced EMT. (B) Cell Viability in Presence of IRI, 5-FU, or OXA for Control HT29 Cell Line and HT29 Cells After EMT Induction. (C) IC_{50} Values for Control HT29 Cells and HT29 Cells With Induced EMT. Green (FITC) Indicates E-Cadherin Expression; Blue (DAPI), Nuclei Staining; and Red (Alexa Fluor 594), Ki-67 Marker. Scale Bar, 100 μm



Abbreviations: DAPI = 4′,6-diamidino-2-phenylindole; EMT = epithelial—mesenchymal transition; 5-FU = 5-fluorouracil; FITC = fluorescein isothiocyanate; IC_{50} = drug concentration causing 50% inhibition; IRI = irinotecan; OXA = oxaliplatin.

Table 1 Clinicopathologic Characteristics of Surgical Specimens Included in Chemosensitivity and Epithelial–Mesenchymal Transition Analyses

Primary Cell Culture	Tumor Site	Tumor Type	Tumor Stage	Differentiation
Col-12	Primary, colon	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-25	Primary, right colon	Adenocarcinoma	T3N0M1a (stage IVA)	Moderate
Col-26	Primary, right colon	Adenocarcinoma	T4bN0M1a (stage IVA)	Moderate
Col-28	Primary, sigmoid	Adenocarcinoma	T4aN2bM1b (stage IVB)	Moderate
Col-29	Primary, sigmoid	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-31	Primary, colon	Adenocarcinoma	T3N1M0 (stage IIIB)	Moderate
Col-32	Primary, colon	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-33	Primary, right colon	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-34	Primary, colon	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-lm-8	Liver metastases	Adenocarcinoma	T3N0M1a (stage IVA)	Moderate
Col-lm-15	Liver metastases	Adenocarcinoma	T4N2M1a (stage IVA)	Moderate
Col-lm-18	Liver metastases	Adenocarcinoma	T3N0M0 (stage IIA)	Moderate
Col-lm-19	Liver metastases	Adenocarcinoma	T3N0M1a (stage IVA)	Moderate
Col-lm-23	Liver metastases	Adenocarcinoma	T4N1M1b (stage IVB)	Moderate

EMT-induced HT29 cells underwent morphologic changes typical of a migration phenotype. They became more elongated, flattened, and loosely adherent to each other, in contrast to the control colonies, which remained compact and consisted of round cells. We suggest that this is due to the loss of E-cadherin expression on the membrane surfaces of these cells. Redistribution of E-cadherin to the nuclear or cytoplasmic regions is clearly seen on ICC images of HT29-EMT cells (Figure 2A). Western blot analysis demonstrated no significant difference in total E-cadherin content and slightly increased level of vimentin after EMT induction (Figure 1B). The proliferative ability of the EMT-induced cells was 2.3 times higher than in the control HT29 cultures according to immunostaining to Ki-67, a nuclear nonhistone protein associated with cell proliferation.[30]

The wound-healing cell migration assay showed that in EMT-induced HT29 cells, the wound closed more rapidly—within 72 hours. In the control HT29 cells, the wound had not recovered by 120 hours. It was found that cancer cells with induced EMT have increased chemosensitivity (Figure 2B). The IC_{50} values in EMT-induced HT29 cells were 128.4 ± 24.8 μM for 5-FU, 11.0 ± 2.4 μM for irinotecan, and 2.6 ± 0.3 μM for oxaliplatin, which is 1.5-, 2-, and 1.8-fold less, respectively, than in the control HT29 cells.

Patient Samples Characterization and Clinical Data

Sixty-three freshly resected surgical specimens from primary colorectal tumors and associated liver metastases were used for the direct establishment of short-term cell cultures in vitro. Approximately 80% of the cultures (51/63) initiated from those specimens were able to proliferate up to the third passage, with the percentage of live cells > 70% (trypan blue staining), while only 22% specimens (14/63) gave primary cell cultures with sufficient quantity of cells to perform chemosensitivity assay for 3 drugs. Nine of the 14 specimens were from primary tumors, and 5 were from liver metastases. Patients had disease stage II to IV, and all the tumors were moderately differentiated. Clinicopathologic data are listed in Table 1.

Originally, short-term cultures were established as suspension spheroid-formed cultures weakly adherent to the plastic surface. For the MTT assays and the ICC studies, the cells were attached to collagen-coated plates to simplify and improve the accuracy of the analyses. Representative images of the short-term cell cultures are presented in Figure 3. The tumor origin of all the short-term cultures was verified by their expression of the epithelial marker EpCam.

E-Cadherin Expression in Patient Samples

The E-cadherin expression was analyzed in each primary cell culture. It was found that 9 of the 14 primary cell cultures (6 lines isolated from primary tumors and 3 from metastases) displayed E-cadherin–positive staining, while the rest, 5 cell cultures (3 from primary tumors and 2 from metastases), were negative for E-cadherin staining (Table 2, Supplemental Table 1 in the online version).

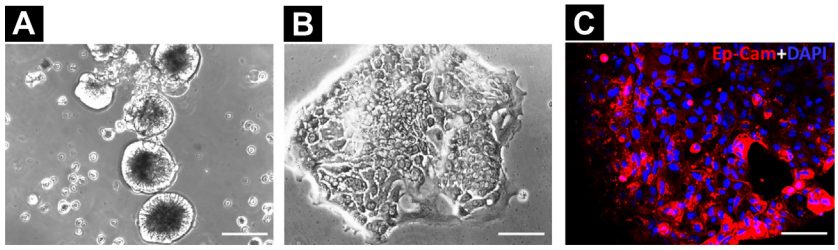
To verify that the isolated cancer cells were similar to the parental tumors in E-cadherin expression, an IHC investigation of the tumor samples was performed (Figure 4). The coincidence of E-cadherin expression in the parental tumors and short-term cell cultures was revealed in most cases (Table 2).

Correlation of Chemosensitivity With E-Cadherin Expression in Short-Term Primary Cell Cultures

To characterize chemosensitivity of patient cancer cells and correlate it with E-cadherin expression, the viability of 14 short-term primary cell cultures exposed to oxaliplatin, 5-FU, and irinotecan was assessed by MTT assay. Chemotherapeutic drugs were added to the cells at IC_{50} values established for the HCT116 (Figure 5) and HT29 (Supplemental Figure 1 in the online version) cell lines.

It should be noted that E-cadherin–positive short-term cultures were less sensitive to all the drugs tested (cell viability was above 56% when IC_{50} doses of HCT116 were used) compared to E-cadherin–negative cultures. Each of the E-cadherin–negative cultures showed sensitivity to at least one drug, and only one culture (Col-25) was sensitive to all 3 drugs.

Figure 3 Representative Images of Short-Term Primary Cell Cultures. (A) Phase-Contrast Microphotograph of Suspension Colonospheres. (B) Phase-Contrast Microphotographs of Adherent Culture. (C) Verification of Tumor Origin of Cells Using Immunofluorescent Staining for Epithelial Marker EpCam and Antibodies Conjugated With Fluorescent Marker Alexa Fluor 594 (Red Color). DAPI (Blue Color) Was Used for Nuclear Staining. Scale bar, 100 μm



Abbreviation: DAPI = 4′,6-diamidino-2-phenylindole.

The statistically significant dependency of cells viability ($P < .05$) on the E-cadherin expression was demonstrated for all 3 drugs tested.

The results of chemosensitivity assessment and E-cadherin expression analysis in short-term primary cell cultures are summarized in the Table 2.

Therefore, the tendency for decreased chemosensitivity in short-term primary cell cultures with non-EMT phenotype was observed. However, further investigations must be performed to clarify the mechanisms of chemosensitivity with regard to the mechanisms of each drug action, taking into account the individual characteristics of each tumor.

Discussion

Recently it has been postulated that EMT is not obligatory for metastasis and is crucial for chemoresistance in non—small-cell lung cancer and pancreatic cancer.[12,19] Likewise, the role of the key EMT marker, E-cadherin, in the chemosensitivity of different cancer types has been widely investigated during the last decade. The pooled data are controversial and were obtained either in vitro for specific cell cultures or during retrospective investigation of patient samples. Only one study has been performed in isolated cancer cells from tumors of patients with triple-negative breast cancer.[31] It is apparent that complex analysis involving several experimental systems is required to elucidate the link between the sensitivity of cancer to chemotherapy and EMT.

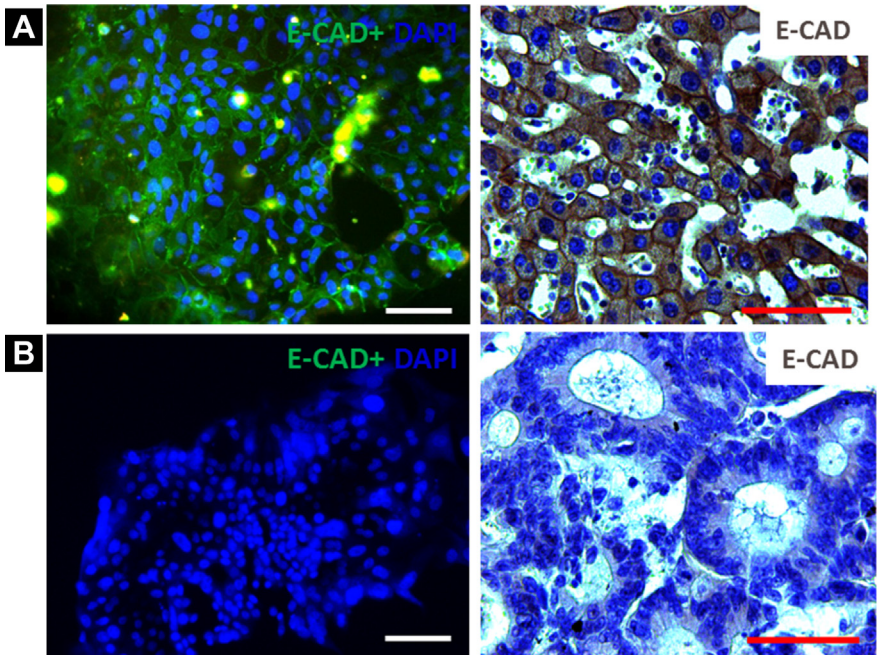
Here we analyzed the innate chemosensitivity of short-term primary cell cultures, isolated from patients' colorectal tumors, in parallel with their E-cadherin expression. Three major agents used in the conventional chemotherapy of CRC were investigated: irinotecan, oxaliplatin, and 5-FU. IHC staining was performed to verify the identity of the short-term primary cell cultures. To our knowledge, such extensive analysis of E-cadherin expression and chemosensitivity has never previously been carried out on primary patient-derived cell cultures.

Table 2 E-Cadherin Expression and Chemosensitivity of Short-Term Primary Cell Cultures at IC_{50} Values Established for HCT116

Primary Cell Culture	E-Cadherin Expression		Viability (%)	Viability (%)	Viability (%)
	ICC	IHC[a]	IRI	5-FU	OXA
Col-12	Positive	1	56.8 ± 18.1	79.8 ± 21.7	73.2 ± 13.6
Col-29	Positive	4	86.5 ± 17.3	76.5 ± 12.2	80.0 ± 9.3
Col-31	Positive	4	99.9 ± 13.2	70.4 ± 7.9	97.7 ± 16.5
Col-32	Positive	4	85.5 ± 9.5	79.2 ± 14.2	82.5 ± 12.4
Col-33	Positive	3	58.3 ± 16.1	84.4 ± 12.5	78.4 ± 10.8
Col-34	Positive	2	96.4 ± 17.1	59.5 ± 14.6	72.9 ± 12.8
Col-lm-15	Positive	4	87.1 ± 14.4	96.7 ± 11.0	76.0 ± 5.9
Col-lm-23	Positive	4	85.3 ± 12.3	85.3 ± 12.3	85.3 ± 12.3
Col-lm-19	Positive	2	60.7 ± 17.5	80.5 ± 11.1	96.4 ± 18.2
Col-25	Negative	2	48.9 ± 4.4	47.1 ± 8.0	20.0 ± 9.5
Col-26	Negative	0	54.1 ± 9.4	36.2 ± 3.9	90.3 ± 11.0
Col-28	Negative	0	49.7 ± 14.9	37.5 ± 10.6	63.6 ± 20.0
Col-lm-18	Negative	0	81.6 ± 12.9	81.1 ± 10.2	46.1 ± 5.7
Col-lm-8	Negative	3	38.0 ± 6.1	48.2 ± 3.8	64.6 ± 9.9

Abbreviations: 5-FU = 5-fluorouracil; IC_{50} = drug concentration causing 50% inhibition; ICC = immunocytochemistry; IHC = immunohistochemistry; IRI = irinotecan; OXA = oxaliplatin.
[a]Score for immunoreactivity from 0 to 4, where 0 is ≤ 10%, 1 is 11-30%, 2 is 31-60%, 3 is 60-80%, and 4 is > 80% stained cells.

Figure 4 E-Cadherin Expression in Short-Term Cell Cultures and Patient Tumors. Representative Images of E-Cadherin Positivity (A) and Negativity (B), With Short-Term Culture (Left) and Parental Tumor Tissue (Right). Green (FITC) Indicates E-Cadherin Expression; Blue (DAPI), Nuclei Staining; and Brown, (Peroxidase Activity), E-Cadherin Expression. Scale bar, 100 μm



Abbreviations: DAPI = 4′,6-diamidino-2-phenylindole; FITC = fluorescein isothiocyanate.

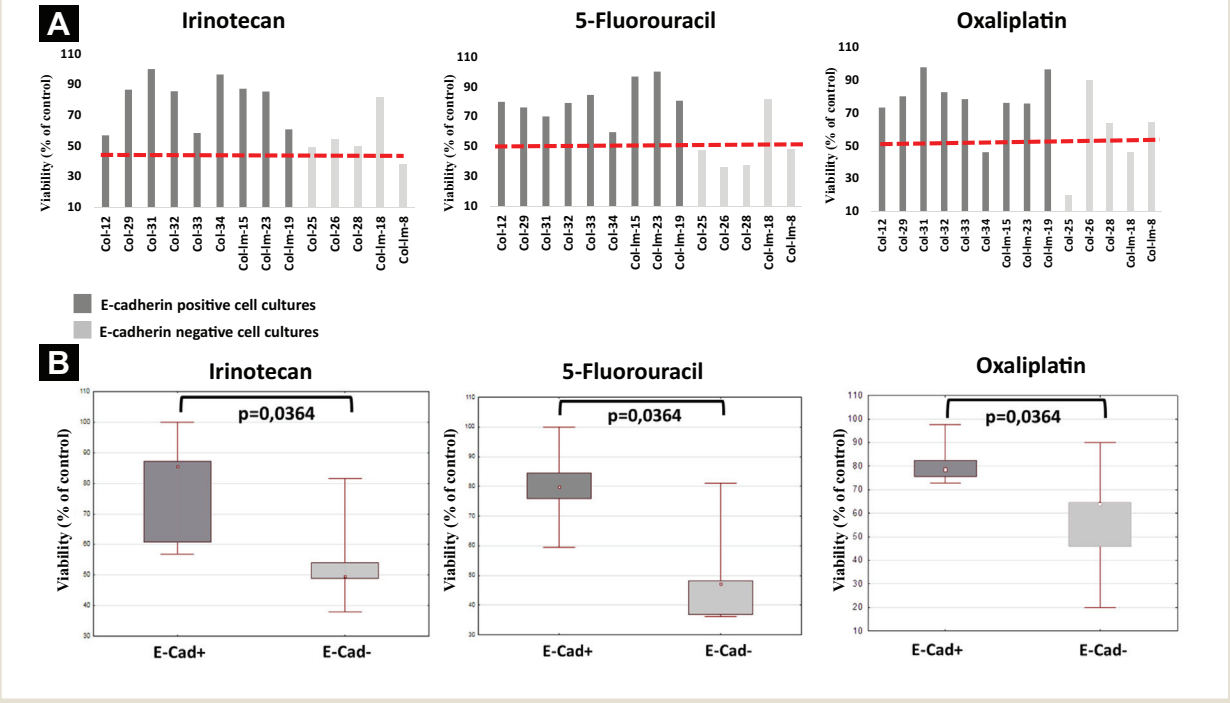
The analysis of chemosensitivity and E-cadherin expression in CRC cell lines showed that the absence of E-cadherin in HCT116 cells correlated with their higher chemosensitivity, whereas HT29 cells with marked membrane E-cadherin expression were more resistant to all the drugs tested. Induction of EMT in the HT29 cell line resulted in a decrease in membrane E-cadherin expression and increased chemosensitivity to all 3 of the chemotherapeutic drugs. Recently a correlation between redistribution of E-cadherin from membranous to cytoplasmic/nuclear localization and cancer stem-cell phenotype in CRC was demonstrated.[32] Moreover, the association of cytoplasmic shift with a higher degree of dysplasia and worsening of prognosis was shown.[32,33] Thus, the redistribution of E-cadherin from cell membrane to the cytoplasm or nucleus and its relation to chemosensitivity can become a subject for further investigation.

In contrast to our results, the majority of studies on cancer cell lines suggest a development of chemoresistance on the induction of EMT via molecular mechanisms such as *Akt* gene suppression, miR-200c and miR-141 down-regulation, *CD44v6* gene up-regulation, or Twist overexpression, or through inducing the gain-of-function mutant p53/ephrin-B2 signaling axis.[12,34-38] However, in most cases, each single molecular mechanism was followed by the development of chemoresistance to a single drug, which suggests that the demonstrated chemoresistance is the result of the modification of a specific pathway rather than the result of EMT. Moreover, such manipulations have sometimes resulted in unexpected cell behavior, particularly a decrease in the metastatic rate,[12] which is atypical of EMT.

To evaluate the role of E-cadherin expression as a predictive marker for clinical use, short-term primary cell cultures from patients' solid tumors were generated because they maintain many of the important markers and functions typical of tumors in vivo. The use of such cell cultures is preferable for personalized medicine.[7,26,39] However, the complexity of their establishment has meant that there is a paucity of such investigations. Nevertheless, the relevance of cell cultures isolated from colorectal tumors as tools to study cancer biology and chemosensitivity has been demonstrated.[26,40] For example, the contribution of the drug-efflux pump ABCB1 to the resistance of colonosphere cultures to irinotecan was revealed. The correspondence of the in vitro chemosensitivity thereof to in vivo xenografts has also been shown.[26] Furthermore, primary cell cultures are an only source of cancer stem cells. Using cancer cells isolated from patients' tumors, the similarity of expression of the cancer stem cell markers (CD44 and CD133) in parent tumor xenografts and in metastases has been demonstrated.[40] Kreso et al[41] showed that inhibition of the canonical self-renewal regulator BMI-1 in cancer stem cells resulted in long-term and irreversible impairment of tumor growth.

In the in vitro study by Koo et al,[31] the increased chemosensitivity of E-cadherin−positive cancer cells isolated from triple-negative

Figure 5 Correlation Between Cell Viability and E-Cadherin Expression in HCT116 Cell Line. (A) Viability of Each Primary Cell Culture Determined by MTT Assay Upon Exposure to Chemotherapeutic Drugs. Cells Were Treated With IC_{50} Values Previously Determined for HCT116 Cell Line. Red Dashed Line Indicates 50% Viability for HCT116 Cells. (B) Dependence of Chemosensitivity on E-Cadherin Expression; Data Presented as Box-and-Whisker Plots. Statistical Analysis Was Carried out by Nonparametric Kolmogorov-Smirnov Test



Abbreviations: IC_{50} = drug concentration causing 50% inhibition; MTT = (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

breast cancer to a wide range of drugs was demonstrated. The expression of cell markers was assessed via IHC in corresponding tumor samples. It was speculated that E-cadherin and the androgen receptors could be predictive factors for the chemotherapy response in triple-negative breast cancer.

In the present study, we analyzed for the first time the chemosensitivity and E-cadherin expression in 14 short-term primary cell cultures directly isolated from solid colorectal tumors (both primary and metastatic). The significant difference of chemosensitivity to oxaliplatin, 5-FU, and irinotecan between E-cadherin−negative and E-cadherin−positive cell cultures was demonstrated. All E-cadherin−positive cultures (9/14) were more resistant, while E-cadherin−negative cultures (5/14) displayed sensitivity to at least 1 of 3 drugs. Obviously, further detailed analysis of E-cadherin expression and other EMT markers using immunoblotting as well as exploration of survival and proliferative pathways are required to explain the differences in chemosensitivity of E-cadherin−negative (according to IHC) cultures. In general, our results are consistent with the studies by Nakamura et al[18] and St Croix et al,[23] where cell−cell adhesion-dependent chemoresistance in relation to conventional chemotherapy, caused predominantly by E-cadherin, was demonstrated in 2-D and 3-D colon immortal cancer cell lines. However, a retrospective study by Nakamoto et al[6] also demonstrated that the expression of E-cadherin may be a positive predictor for cetuximab-based therapy, especially in *KRAS* wild-type colorectal tumors. Only paraffin-embedded samples were analyzed in that study.

The ability of primary cell cultures to recapitulate the gene expression profile of cancer cells in original patient tumors has been widely shown. Many studies report a strong similarity between the primary cell cultures of CRC, patient-derived xenografts, and corresponding patient tumors.[42-44] Specifically, Rajcevic et al[43] demonstrated that epithelial markers such as E-cadherin and EpCam appear to be relatively unchanged in patient-derived CRC spheroids compared to biopsy samples. In our study, we compared the expression of E-cadherin in short-term cell cultures and parental tumors using immunostaining. While we found good concordance between E-cadherin−positive samples, 2 of 5 E-cadherin−negative cultures had a high E-cadherin expression in IHC (score 2 and 3). We speculate that these discrepancies may be explained either by tumor heterogeneity (it is possible that the samples for cell culture establishment and histological analysis were taken from different zones of tumor) or by false-positive staining in IHC.

Although the number of samples we assessed is small for statistical interpretation and we cannot correlate our finding with clinical outcomes, as this was outside the scope of this study, our results suggest that the presence of E-cadherin in cancer cells isolated from patient tumors can be a potential indicator of low chemosensitivity. Further investigations are needed to determine any accompanying

alterations or underlying discrepancies in the chemosensitivity to different drugs occurring during the development of EMT. Plasmid transfection along with short hairpin RNA experiments to achieve overexpression or down-regulation of EMT markers would be useful to further test the results. Moreover, extensive clinical studies with appropriate experimental groups could shed light on the potential use of E-cadherin detection via IHC as a prognostic factor in conventional chemotherapy. In general, the data obtained may serve as a basis for the analysis of colon cancer chemosensitivity using short-term cultures and the assay of E-cadherin expression.

Clinical Practice Points

- During the last decade, several studies have investigated the EMT as the cause of chemoresistance, and the contradictory data have been reported.
- Previously studies reported that E-cadherin acts as a chemoprotective agent by blocking drug diffusion through the intercellular junctions. However, this contradicts the decreased chemosensitivity in cells evidencing EMT.
- Here we report for what is to our knowledge the first time the increasing of chemosensitivity during EMT induction in standard cell culture HT29.
- Data from 14 patient samples revealed chemosensitivity based on primary short-term cell culture analysis; E-cadherin expression was assessed in primary short-term cell cultures and in histologic samples.
- Results demonstrated the negative prognostic effect of E-cadherin in chemotherapy with oxaliplatin, irinotecan, and 5-FU.
- Our data may serve as a basis for the analysis of colon cancer chemosensitivity using short-term cultures and the assay of E-cadherin expression in clinical practice.

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Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

A supplemental figure and table accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2018.10.003.

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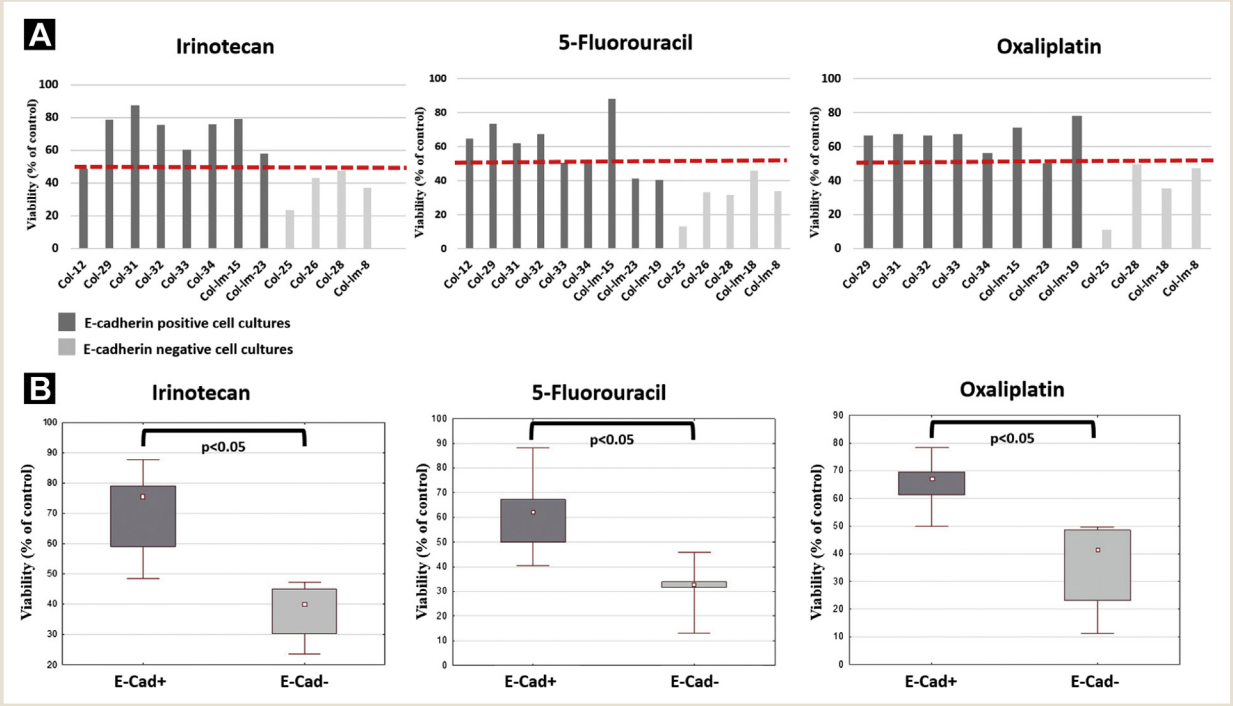
Supplemental Table 1 E-Cadherin Expression and Chemosensitivity of Short-Term Primary Cell Cultures at IC_{50} Values Established for HT29

Primary Cell Culture	E-Cadherin Expression		Viability (%)	Viability (%)	Viability (%)
	ICC	IHC[a]	IRI	5-FU	OXA
Col-12	Positive	1	48.6 ± 9.2	64.6 ± 8.6	—
Col-29	Positive	4	78.8 ± 13.9	73.6 ± 9.4	66.5 ± 10.5
Col-31	Positive	4	87.6 ± 16.0	61.9 ± 15.5	67.2 ± 16.1
Col-32	Positive	4	75.3 ± 13.8	67.2 ± 14.7	66.4 ± 12.5
Col-33	Positive	3	60.2 ± 13.9	50.3 ± 17.0	67.4 ± 14.5
Col-34	Positive	2	75.7 ± 11.5	50.0 ± 7.2	56.3 ± 16.1
Col-lm-15	Positive	4	79.1 ± 8.5	88.1 ± 12.3	71.4 ± 5.6
Col-lm-23	Positive	4	58.0 ± 12.0	41.2 ± 12.3	50.0 ± 16.8
Col-lm-19	Positive	2	—	40.4 ± 15.6	78.3 ± 12.0
Col-25	Negative	2	23.5 ± 6.1	12.9 ± 4.9	11.1 ± 5.4
Col-26	Negative	0	42.9 ± 13.9	32.9 ± 8.5	—
Col-28	Negative	0	47.4 ± 8.7	31.6 ± 12.0	49.7 ± 17.8
Col-lm-18	Negative	0	—	45.9 ± 19.2	35.3 ± 7.6
Col-lm-8	Negative	3	37.0 ± 6.2	33.9 ± 3.5	47.3 ± 5.1

Abbreviations: 5-FU = 5-fluorouracil; IC_{50} = drug concentration causing 50% inhibition; ICC = immunocytochemistry; IHC = immunohistochemistry; IRI = irinotecan; OXA = oxaliplatin.
[a]Score for immunoreactivity from 0 to 4, where 0 is $\leq$ 10%, 1 is 11-30%, 2 is 31-60%, 3 is 60-80%, and 4 is $>$ 80% stained cells.

Supplemental Figure 1 Correlation Between Cell Viability and E-Cadherin Expression in HT29 Cell Line. (A) Viability of Each Primary Cell Culture Determined by MTT Assay Upon Exposure to Chemotherapeutic Drugs. Cells Were Treated With IC_{50} Values Previously Determined for HT29 Cell Line. Red Dashed Line Indicates 50% Viability for HCT116 Cells. (B) Dependence of Chemosensitivity on E-Cadherin Expression; Data Presented as Box-and-Whisker Plots. Statistical Analysis was Carried out by Nonparametric Kolmogorov-Smirnov Test



Abbreviations: IC_{50} = drug concentration causing 50% inhibition; MTT = (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.