

Chronic Oxaliplatin Resistance Induces Epithelial-to-Mesenchymal Transition in Colorectal Cancer Cell Lines

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Abstract Purpose: Epithelial-to-mesenchymal transition (EMT) is a process whereby cells acquire molecular alterations that facilitate cell motility and invasion. In preliminary studies, we observed that oxaliplatin-resistant (OxR) colorectal cancer (CRC) cells underwent morphologic changes suggestive of a migratory phenotype, leading us to hypothesize that OxR CRC cells undergo EMT.

Experimental Design: The human CRC cell lines KM12L4 and HT29 were exposed to increasing doses of oxaliplatin to establish stable cell lines resistant to oxaliplatin. Migration and invasion were assessed by modified Boyden chamber assays. Morphologic and molecular changes characteristic of EMT were determined by immunofluorescence staining and Western blot analyses.

Results: The OxR cells showed phenotypic changes consistent with EMT: spindle-cell shape, loss of polarity, intercellular separation, and pseudopodia formation. KM12L4 and HT29 OxR cells exhibited an ~8- to 15-fold increase in migrating and invading cells, respectively ($P < 0.005$ for both). Immunofluorescence staining of OxR cells revealed translocation of E-cadherin and β -catenin from their usual membrane-bound complex to the cytoplasm and nucleus, respectively. The OxR cells also had decreased expression of the epithelial adhesion molecules E-cadherin and plakoglobin and an increase in the mesenchymal marker vimentin. The KM12L4 OxR cells exhibited increased nuclear expression of Snail, an EMT-regulatory transcription factor, whereas the HT29 OxR cells exhibited an increase in nuclear expression of the EMT-associated transcription factor nuclear factor κ B.

Conclusion: We hypothesize that induction of EMT may contribute to the decreased efficacy of therapy in chemoresistant CRC, as the tumor cells switch from a proliferative to invasive phenotype. Further understanding of the mechanisms of chemoresistance in CRC will enable improvements in chemotherapy for metastatic disease.

Oxaliplatin is a third-generation platinum compound and is the first platinum-based compound to show efficacy in the treatment of colorectal cancer (CRC; ref. 1). Its use in combination with 5-fluorouracil and leucovorin (FOLFOX) for metastatic CRC has led to response rates >50% and median survival approaching 2 years (2, 3). FOLFOX has also been

found to be very effective in the adjuvant setting, leading to an increase in the number of patients who are cured after surgical resection when compared with the use of 5-fluorouracil and leucovorin alone (4). Despite these impressive accomplishments, virtually all metastatic CRC eventually become resistant to oxaliplatin, with a median time to progression of ~8 months (5). Hypotheses on the mechanisms of oxaliplatin resistance include defects in oxaliplatin uptake, impaired DNA adduct formation, and increased expression of a copper efflux transporter (6–9).

Epithelial-to-mesenchymal transition (EMT) is a process initially observed in embryonic development in which cells lose epithelial characteristics and gain mesenchymal properties to increase motility and invasion (10). Previous research suggests that EMT is also important in tumor progression and metastasis (10, 11) and is induced by growth factors implicated in these processes such as hepatocyte growth factor, transforming growth factor β , and epidermal growth factor (12).

In preliminary studies, our laboratory observed that oxaliplatin-resistant (OxR) CRC cells exhibit an altered phenotype whereby cells disperse, develop pseudopodia, and assume a spindle shape, properties associated with the EMT phenotype. Based on the above observations, we hypothesized

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Note: A.D. Yang and F. Fan contributed equally to this work.

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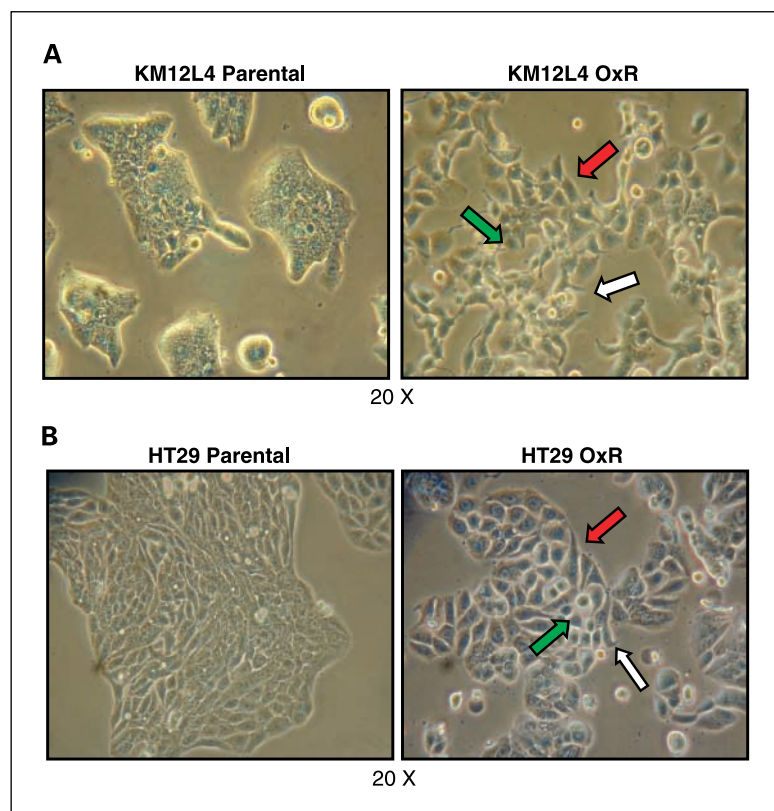


Fig. 1. Acquisition of oxaliplatin resistance induces morphologic changes consistent with EMT in CRC cells. KM12L4 (A) and HT29 (B) parental and OxR cells were assessed for morphologic changes consistent with EMT. Spindle-shaped cells with loss of polarity (red arrows), increased intercellular separation (green arrows), and pseudopodia (white arrows) were noted in the OxR cells but not in parental cells from both cell lines.

that oxaliplatin resistance leads to an EMT phenotype, including its characteristic molecular alterations. To test this hypothesis, we assessed OxR cells derived from two human CRC cell lines for gross morphologic, immunohistochemical, and molecular changes consistent with EMT.

Materials and Methods

Cell lines and culture conditions. The human CRC cell line KM12L4 was obtained from I.J. Fidler, D.V.M., Ph.D. (The University of Texas M.D. Anderson Cancer Center, Houston, TX). The human CRC cell line HT29 was obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in MEM supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids (Life Technologies, Grand Island, NY), and HEPES buffer (MP Biomedicals, Irvine, CA) at 37°C in 5% CO₂ and 95% air. Cells were confirmed to be free of *Mycoplasma* using a Mycoplasma Detection Kit (American Type Culture Collection). *In vitro* experiments were done at 50% to 70% cell confluence. Results from all studies were confirmed in at least three independent experiments.

Drugs and antibodies. Oxaliplatin (Sanofi-Synthelabo, New York, NY) was purchased from the pharmacy at M.D. Anderson Cancer Center. Antibodies used for immunofluorescence staining and Western blot analyses were as follows: mouse anti-E-cadherin (Zymed Laboratories, Carlsbad, CA), mouse anti-plakoglobin, mouse anti-vimentin, mouse anti-smooth muscle actin, mouse anti-fibronectin (Chemicon International, Temecula, CA), mouse anti-N-cadherin, rabbit anti-β-catenin (Upstate Chemical, Temecula, CA), rabbit anti-Snail, rabbit anti-Twist, goat anti-Slug, rabbit anti-nuclear factor κB (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-actin, mouse anti-vinculin

(Sigma-Aldrich, St. Louis, MO), and rabbit anti-lamin B1 (Active Motif, Carlsbad, CA).

Development of OxR CRC cell lines. To create stable CRC cell lines chronically resistant to oxaliplatin, KM12L4 and HT29 cells were exposed to an initial oxaliplatin concentration of 0.1 μmol/L in MEM plus 10% FBS. The surviving population of cells was grown to 80% confluence and passaged twice over 9 days to ensure viability. The concentration of oxaliplatin the surviving population was exposed to was then sequentially increased in the same manner to 0.5 μmol/L (15 days), 1.0 μmol/L (30 days), and finally to the clinically relevant plasma concentration of 2 μmol/L. For all *in vitro* studies, each OxR cell line was used at no higher than 15 passages from creation. To confirm that the OxR cell lines would remain resistant *in vivo*, 2 million cells were injected s.c. into athymic nude mice as previously described (13). Tumors were harvested after ~30 days and tumor cells were replated and exposed to 2 μmol/L oxaliplatin.

Morphologic analysis. Cells were grown to 70% confluence in MEM plus 10% FBS (parental cell lines) or MEM plus 10% FBS plus 2 μmol/L oxaliplatin (OxR cell lines) and visualized at ×20 magnification with a Nikon light microscope (Nikon, Inc., Melville, NY) with digital photographic capability. The digital images of the OxR cells and parental cells were compared for morphologic characteristics consistent with EMT [i.e., loss of polarity (spindle-shaped cells), increase in intercellular separation, and appearance of pseudopodia]. Blinded observers classified the images with regard to presence or absence of morphologic changes consistent with EMT.

Migration and invasion assays. Cell migration and invasion were assessed with Boyden chambers or modified Boyden chambers according to the protocol of the manufacturer (Becton Dickinson Labware, Bedford, MA). Briefly, 100,000 KM12L4 parental and OxR cells in MEM plus 1% FBS with or without 2 μmol/L oxaliplatin were placed on each 8.0-μm pore size membrane insert in 24-well plates. MEM plus 10% FBS, with or without 2 μmol/L oxaliplatin, was placed

in the bottom wells as chemoattractants. After 24 hours, cells that did not migrate were removed from the top side of the inserts with a cotton swab. Cells that had migrated to the underside of the inserts were stained with Diff-Quik (Harleco, Gibbstown, NJ) and the cells on each insert were counted at $\times 100$ magnification. The invasion assay was done in a similar fashion except the 8.0- μm pore size membrane inserts were coated with Matrigel. Results were expressed as mean \pm SE.

Fluorescent immunohistochemistry. KM12L4 and HT29 parental and OxR cells were grown on poly-L-lysine-coated glass coverslips (BD Biosciences, San Jose, CA) to 40% to 50% confluence; after 48 hours of incubation, the cells were fixed with cold acetone for 1 hour and permeabilized in 0.5% Triton X-100 (Sigma-Aldrich) for 10 minutes. The cells were then blocked with normal horse and goat serum in PBS. Cells were incubated with primary antibodies (anti-E-cadherin or anti- β -catenin) overnight at 4°C. The following morning, the slides were washed with PBS and incubated with the appropriate FITC-conjugated secondary antibody for 1 hour. The cells were then washed and incubated with Hoechst 33342 (Invitrogen Corp., Carlsbad, CA) for nuclear staining, washed, and mounted with propyl gallate under glass coverslips. The slides were visualized for immunofluorescence with a laser scanning Olympus microscope (Olympus America, Inc., Melville, NY).

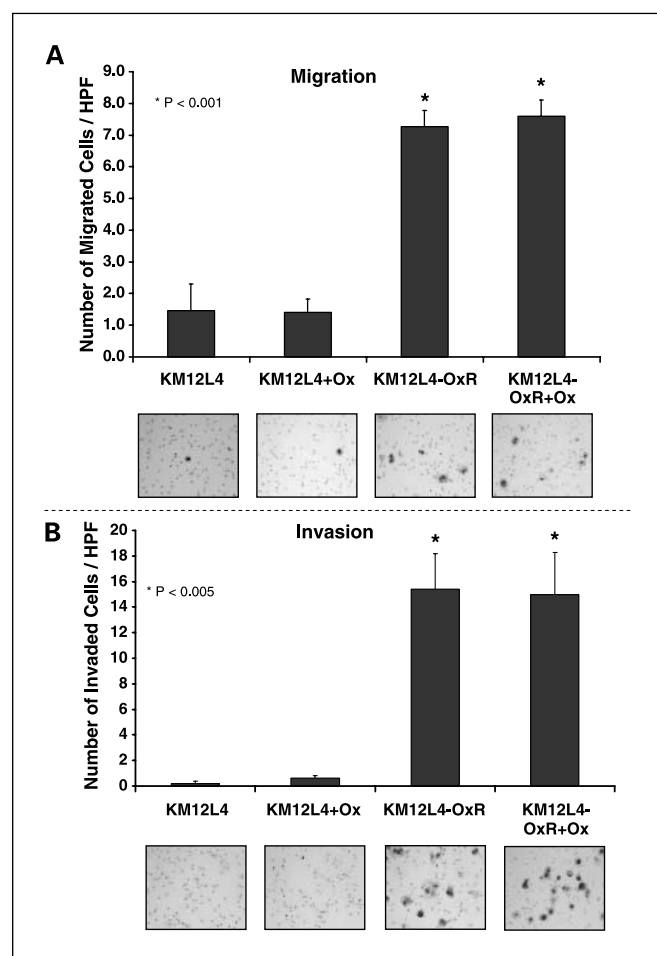


Fig. 2. OxR CRC cells have increased migratory and invasive capacity. Boyden chamber and modified Boyden chamber assays were done to compare the migratory and invasive capabilities of KM12L4 OxR and parental cells. *A*, at 48 hours, the OxR cells showed an ~ 7.5 -fold increase in the number of cells migrating through the collagen insert. *B*, also at 48 hours, the OxR cells exhibited an ~ 15 -fold increase in the number of cells invading through the Matrigel-coated collagen insert. *HPF*, high-power field; *Ox*, oxaliplatin.

Western blot analysis. For protein extraction, KM12L4 and HT29 parental and OxR cells were plated and grown to 70% to 80% confluence. Whole-cell protein was isolated using radioimmunoprecipitation assay B protein lysis buffer as previously described (14). Nuclear protein was extracted with a commercially available kit (Active Motif). The isolated protein was quantified by a commercially available modified Bradford assay (Bio-Rad Laboratories, Hercules, CA). Western blot protein samples were prepared by boiling the isolated protein with denaturing sample buffer. The protein was then separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membranes were blocked with 5% nonfat dry milk in TBS and 0.1% Tween 20 for 1 hour and probed with the appropriate primary antibody overnight at 4°C. The next morning, the membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature. The membranes were then washed and protein bands visualized by using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). To verify the accuracy of whole-cell lysate and nuclear extract protein loading, membranes were incubated in stripping solution for 30 minutes at 65°C, washed, and reprobed with β -actin, vinculin, or lamin-B1 antibody as a loading control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. KM12L4 and HT29 parental and OxR cells were plated in 96-well plates at 4,000 per well with MEM plus 10% FBS and incubated for 24, 48, or 72 hours. At the end of the incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added to achieve a final concentration of 0.5 mg/mL and the cells were incubated for another 60 minutes. Medium and MTT were then removed, DMSO was added for 1 minute to induce cell lysis, and absorption was read at 570 nm.

Flow cytometry and cell cycle analysis. KM12L4 and HT29 parental and OxR cells were grown to 70% to 80% confluence in MEM plus 10% FBS (parental cell lines) or MEM plus 10% FBS plus 2 $\mu\text{mol/L}$ oxaliplatin (OxR cell lines) over 48 hours. After trypsinization, cells were washed in PBS and fixed in 70% ethanol at 4°C for 2 hours. DNA staining was done with 10 mg propidium iodide/mL PBS and 2.5 μg DNase-free RNase (Roche Diagnostics)/mL PBS for at least 30 minutes before flow cytometry in a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Cell cycle profiles were generated from flow cytometry analysis with MultiCycle software (Phoenix Flow Systems, San Diego, CA).

Statistical analysis. Student's *t* test was used in all statistical analyses of MTT, invasion, and migration assay results using InStat Statistical Software version 2.03 (GraphPad Software, San Diego, CA). Statistical significance was defined as two-tailed $P \leq 0.05$.

Results

Acquisition of oxaliplatin resistance induces morphologic changes consistent with EMT in CRC cells. We first noted that cells from the human KM12L4 and HT29 CRC cell lines that had acquired resistance to oxaliplatin at the clinically relevant concentration of 2 $\mu\text{mol/L}$ had a markedly different light-microscopic appearance from cells of the parental cell lines. The phenotypic changes observed in OxR cells included loss of cell polarity causing a spindle-cell morphology, increased intercellular separation signifying loss of intercellular adhesion, and increased formation of pseudopodia (Fig. 1). These changes are typical of cells with a mesenchymal phenotype.

OxR CRC cells have increased migratory and invasive capacity. Boyden chamber assays were done to compare the migratory capability of KM12L4 OxR and parental cells. At

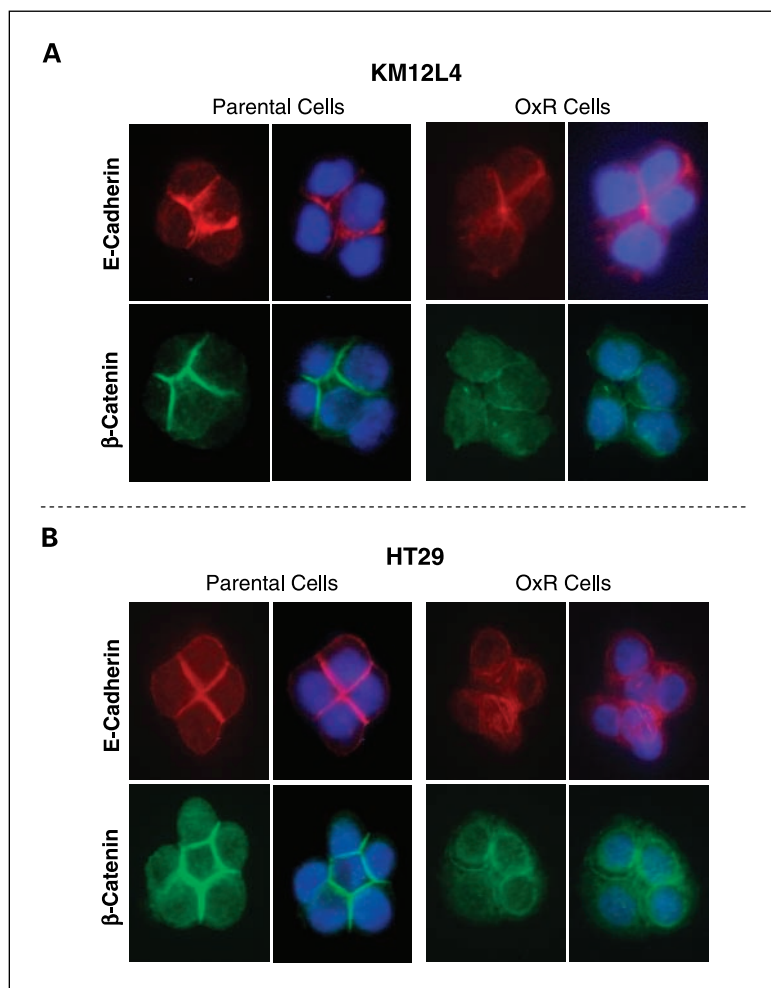


Fig. 3. OxR CRC cells exhibit changes in localization of cellular EMT markers. Immunofluorescence staining for E-cadherin and β -catenin was done on KM12L4 (A) and HT29 (B) parental and OxR cells. OxR cells from both cell lines showed changes in localization of E-cadherin and β -catenin from their usual cell membrane-associated site. OxR cells exhibited E-cadherin in a disorganized cytoplasmic location and β -catenin was noted to translocate to the nucleus.

48 hours, the OxR cells showed an ~ 7.5 -fold increase in the number of cells migrating through the collagen membrane ($P < 0.001$; Fig. 2A). The capacity of OxR cells to invade through a Matrigel-coated membrane was even greater, with an ~ 15 -fold increase in the number of invading OxR cells compared with parental cells ($P < 0.005$; Fig. 2B). The presence or absence of $2 \mu\text{mol/L}$ oxaliplatin in the cell culture medium did not significantly affect the migratory or invasive capacity of the OxR cells.

OxR CRC cells exhibit changes in the localization of cellular E-cadherin and β -catenin. One of the hallmarks of EMT is the breakdown of the cytoplasmic cell adhesion complex (15), which causes a change in the localization of E-cadherin and β -catenin from their usual membrane-bound site. The loss of E-cadherin from the membrane facilitates a loss of homotypic epithelial cell adhesion and releases β -catenin from its cell-surface location. The subsequent translocation of β -catenin to the nucleus allows it to bind to other proteins as part of a transcriptional complex that mediates other cellular alterations associated with EMT (16). To investigate the possibility that oxaliplatin resistance leads to changes in the cellular localization of E-cadherin and β -catenin, immunofluorescence staining was done on KM12L4 (Fig. 3A) and HT29 (Fig. 3B) OxR and parental cells. In both cell lines, the OxR cells exhibited a change in E-cadherin from an organized, membrane-bound

structure to a disorganized state in which it was noted to be dispersed throughout the cytoplasm. Furthermore, β -catenin was observed to translocate from the usual membrane-bound site observed in the parental cells to the nucleus in both OxR cell lines.

OxR CRC cells exhibit molecular changes consistent with EMT. To determine if the acquisition of oxaliplatin resistance induced the specific molecular changes consistent with EMT, Western blotting was done on cell lysates and nuclear extracts from the KM12L4 OxR and parental cells (Fig. 4A). Expression of the epithelial adhesion molecules E-cadherin and plakoglobin was decreased in the OxR cells compared with the parental cells. A concurrent marked increase in the expression of the mesenchymal marker vimentin was also observed. There was no change in expression of the mesenchymal markers N-cadherin, α -smooth muscle actin, and fibronectin (data not shown). Furthermore, increased nuclear expression of the EMT-related transcription factor Snail was observed in OxR KM12L4 cells compared with the parental cells (15, 17). No significant changes were observed in nuclear levels of the EMT-related transcription factors Slug and Twist (also known as Snail-2 and Snail-3, respectively). Similar results were noted when validation studies were done in HT29 OxR and parental cells for the epithelial markers E-cadherin and plakoglobin. In contrast to the KM12L4 OxR cells, no change in expression of

mesenchymal markers (data not shown) or nuclear expression of Snail, Slug, and Twist was observed in the HT29 OxR cells when compared with the parental cells (Fig. 4B). However, in the HT29 OxR cells, nuclear expression of the transcription factor nuclear factor κ B was found to be increased (Fig. 4B). The expression level of β -catenin was not changed in either the KM12L4 or HT29 OxR cell line when compared with the parental cell lines in whole-cell protein extracts, cytoplasmic protein extracts, or nuclear protein extracts (data not shown).

OxR CRC cells exhibit reduced proliferation rates. The KM12L4 (Fig. 5A) and HT29 (Fig. 5B) parental and OxR cell numbers were compared at different time points after plating by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At 48 and 72 hours, cell counts increased in all groups but both the KM12L4 and HT29 OxR cell lines exhibited reduced cell counts compared with the parental cell lines ($P < 0.005$ for both comparisons). Cell cycle analysis showed an increase in the percentage of cells in G_2 and no change in the percentage of cells undergoing apoptosis (sub- G_0 ; Table 1). This suggests that the finding of decreased cell number in the MTT assay can be attributed to decreased proliferation of the OxR cells.

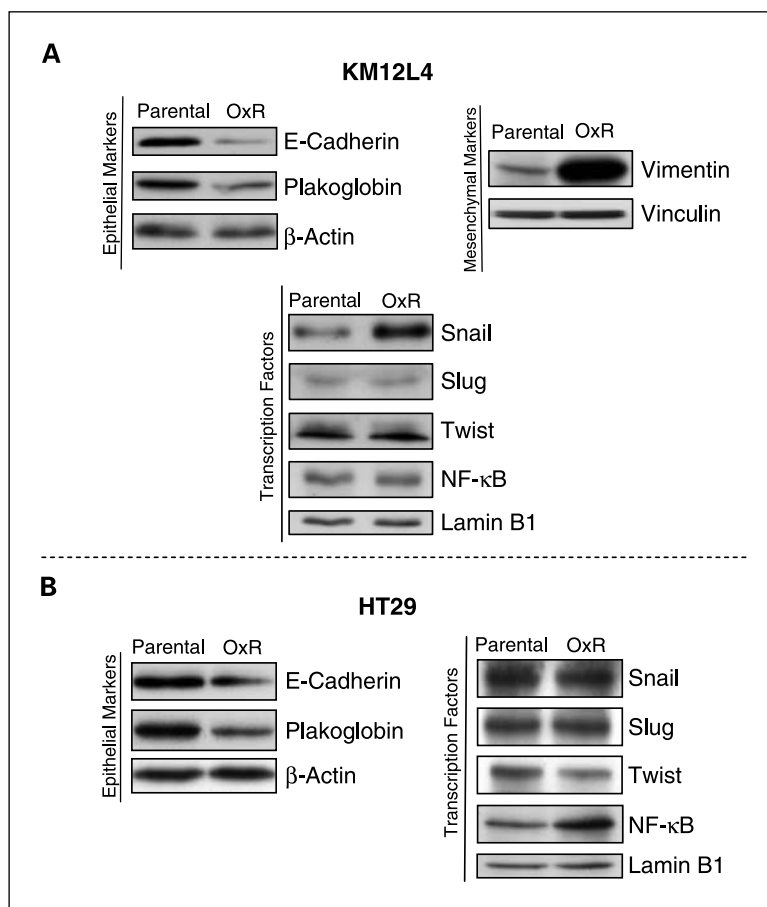
Discussion

The last decade has witnessed major innovations in the treatment of metastatic CRC. The median survival for affected

patients has increased steadily and is now crossing the 2-year barrier (18). The advent of oxaliplatin played a major role in this disease and it is now part of the most commonly used regimens both in the metastatic as well as in the adjuvant setting. However, it is far from being the perfect treatment. Despite the oxaliplatin activity, the average responding patient can expect to develop resistance ~6 to 8 months after the treatment was initiated. Given enough time, the development of resistance is uniform, and rare patients with metastatic disease, if any, are cured without surgery. Cellular levels of glutathione and ERCC-1 seem to be associated with inherent resistance to oxaliplatin and may play a role in the acquired resistance as well (7). Decreased cellular uptake and adduct formation have been shown but the mechanisms of development of resistance during treatment are still poorly understood (8).

We showed in this series of experiments that acquisition of oxaliplatin resistance by CRC cells leads to morphologic and molecular alterations consistent with a change to a mesenchymal-like phenotype. Furthermore, we showed that OxR CRC cell lines had changes in the cellular localization of two major factors associated with EMT, E-cadherin and β -catenin. Although expression of E-cadherin was decreased in both OxR cell lines, we did not observe changes in β -catenin protein expression levels in whole-cell, cytoplasmic, or nuclear extracts. This lack of change in β -catenin expression could be due to high constitutive expression of β -catenin in these cell lines secondary to APC mutation or it may reflect the

Fig. 4. OxR CRC cells exhibit molecular changes consistent with EMT. **A**, cell lysates and nuclear extracts from KM12L4 parental and OxR cells were subjected to Western blotting. Expression of the epithelial cellular adhesion molecules E-cadherin and plakoglobin was decreased in OxR cells compared with parental cells (*top left*). A marked increase in the expression of the mesenchymal marker vimentin was concurrently observed (*top right*). There was also increased expression of the EMT-related transcription factor Snail in nuclear extracts of OxR cells compared with parental cells (*bottom*). No change in expression of the EMT-related transcription factors Slug or Twist was noted. **B**, similar results were noted when validation studies were done in the HT29 parental and OxR cells (*left*) except that no up-regulation of mesenchymal markers was noted and no changes were noted in Snail, Slug, or Twist. However, nuclear expression of another transcription factor implicated in the induction of EMT, nuclear factor κ B (*NF- κ B*), was observed in the HT29 OxR cell line (*right*).



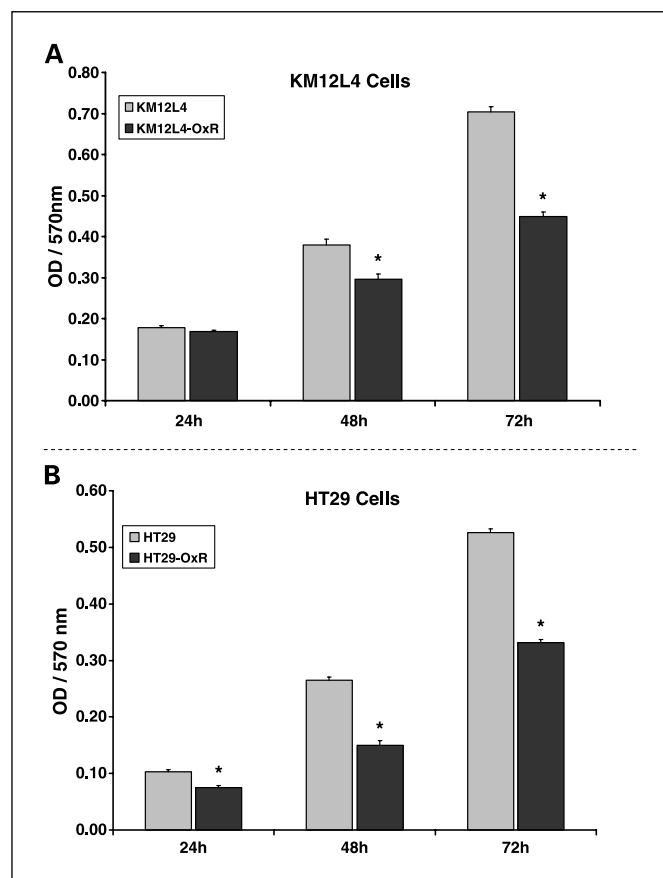


Fig. 5. OxR CRC cells have reduced cell number. The cell number of KM12L4 (A) and HT29 (B) parental and OxR cells were compared by MTT assay. At 24, 48, and 72 hours, both the KM12L4 and HT29 OxR cells exhibited reduced cell number compared with the respective parental cells.

fact that this may be a relocation phenomenon that is not accompanied by changes in β -catenin protein levels. Of note, although we discovered molecular evidence that the EMT changes in the KM12L4-OxR cell line were associated with an increase in the nuclear expression of the transcription factor Snail, we were not able to detect changes in Snail, Slug, or Twist in the HT29 OxR cell line. However, nuclear expression of the transcription factor nuclear factor κ B, which has been shown to be associated with EMT in a mammary carcinoma model (19), was increased in the HT29 OxR cell line only. Given that we were unable to observe an increase in any mesenchymal markers in the HT29 OxR cell line, we postulate that the HT29 OxR cells may be undergoing an

incomplete change to a mesenchymal-like phenotype rather than the full EMT observed in the KM12-OxR cells. This could be explained by the different signaling pathways (Snail versus nuclear factor κ B) inducing the phenotypic change in the two OxR cell lines. Finally, we also showed that the OxR cells undergoing EMT had increased migratory and invasive capabilities. This finding of increased aggressiveness was consistent in two OxR CRC cell lines.

Recent research has implicated EMT in cancer progression by noting that epithelial-derived tumor cells can switch their phenotype to a more primitive mesenchymal phenotype that facilitates motility and invasion (10). Several studies have examined the possible role of EMT in CRC progression (20, 21). Although two studies have shown that loss of E-cadherin-mediated adhesion decreases chemoresistance (22, 23), to our knowledge, no studies have described EMT with loss of E-cadherin-mediated adhesion occurring in cells that have acquired chemoresistance. We believe that our finding that OxR CRC cells undergo epithelial to mesenchymal or mesenchymal-like transition reflects an important process by which cancer cells may potentially acquire chemoresistance. We further hypothesize that OxR cells may switch their "molecular machinery" from a proliferative, epithelial phenotype to a more invasive and migratory mode. Because proliferation is required for oxaliplatin-induced chemosensitivity, the decrease in proliferation of OxR cells may be one means whereby resistant cells can escape the effects of chemotherapy.

In conclusion, this is, to our knowledge, the first description of chemoresistance-induced EMT, and we postulate that EMT induced by acquisition of oxaliplatin resistance could be a possible survival mechanism for chemoresistant CRC cells. If our hypothesis is true, blocking or reversing EMT changes may cause chemoresistant cells to revert to chemosensitive cells. Furthermore, it is yet to be determined if these findings in OxR cells are applicable in cells that develop resistance to other chemotherapeutic drugs. We believe that induction of EMT in chemoresistant CRC represents a new potentially exciting area of research into the mechanism of CRC progression and may eventually be of benefit to patients with advanced, chemoresistant CRC patients who currently do not have many effective treatment options.

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Table 1. Cell cycle analysis of KM12L4 and HT29 OxR cells

48 hours	Sub-G ₀ (%)	G ₁ (%)	S (%)	G ₂ (%)
KM12L4 parental	11	49	38	13
KM12 OxR	8	37	29	34
HT29 parental	3	68	24	8
HT29 OxR	3	53	27	20

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