Chemosensitivity analysis and study of gene resistance on tumors and cancer stem cell isolates from patients with colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the main causes of mortality. Recent studies suggest that cancer stem cells (CSCs) can survive after chemotherapy and promote tumor invasiveness and aggression. According to a higher hierarchy complexity of CSC, different protocols for isolation, expansion, and characterization have been used; however, there are no available resistance biomarkers that allow predicting the clinical response of treatment 5-fluorouracil (5FU) and oxaliplatin. Therefore, the primary aim of the present study was to analyze the expression of gene resistance on tumors and CSC-derived isolates from patients CRC. In the present study, adenocarcinomas of the colon and rectum (CRAC) were classified based on an *in vitro* adenosine triphosphate-based chemotherapy response assay, as sensitive and resistant and the percentage of CD24 and CD44 markers are evaluated by immunohistochemistry. To isolate resistant colon-CSC, adenocarcinoma tissues resistant to 5FU and oxaliplatin were evaluated. Finally, all samples were sequenced using a custom assay with chemoresistance-associated genes to find a candidate gene on resistance colon-CSC. Results showed that 59% of the CRC tissue analyzed was resistant and had a higher percentage of CD44 and CD24 markers. An association was found in the expression of some genes between the tumor-resistant tissue and CSC. Overall, isolates of the CSC population CD44+ resistant to 5FU and oxaliplatin demonstrated different expression profiles; however, the present study was able to identify overexpression of the *KRT-18* gene, in most of the isolates. In conclusion, the results of the present study showed overexpression of *KRT-18* in CD44+ cells is associated with chemoresistance to 5FU and oxaliplatin in CRAC.

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Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide, being the third most commonly diagnosed, and the second most deadly cancer (1,2). Therapeutically, patients with CRC are treated with a combined regimen of 5-fluorouracil (5FU), folinic acid (FA) and oxaliplatin (FOLFOX-6), which have been demonstrated to improve prognosis (3). However, <10% of patients at stage IV survive for >5 years (4,5) and >90% of patients tend to develop metastasis and chemoresistance (6,7). The presence of cancer stem cell (CSC) populations are associated with chemoresistance, as well as with tumor-initiating cells (TICs) (8-10). CSCs are characterized by self-renewal, limitless proliferation and differentiation into various epithelial lineages, and generating tumor heterogeneity (11); mainly derived from genetic alterations, they develop from either intestinal stem cells or from differentiated intestinal cells by epithelial-mesenchymal transition (EMT) (12,13). Specific markers for normal stem

cells, such as Lgr5, CD44, CD24, CD26, CD29, CD166, CD326, CD133, EpCAM and ALDH, are commonly used to isolate and characterize CSCs (14,15). Previous studies suggest that the number of CSCs in tumors and metastasis become enriched after starting chemotherapy and only a small subpopulation survive (16-18). These surviving CSCs expand after chemotherapy; hence it should be no surprise that several mechanisms are involved in the process of chemoresistance and that some specific genes are implicated as potential candidates for resistance to anticancer agents (19-21).

Previous studies have identified response markers that predict potential therapeutic targets and used next generation sequencing to identify sensitivity of these biomarkers, and the genomic and genetic characteristics of cancer cell lines or tumor tissue samples (22-26). The present study analyzed the expression of the genetic profile of colon-CSC CD44$^+$ cells resistant to 5FU and oxaliplatin using a custom assay with chemoresistance genes to discover a candidate gene of colon-CSC.

Materials and methods

Participants. A total of 51 patients with CRC who underwent resection of the primary tumor were enrolled between January 2014 and December 2016 at the Autonomous University of Nuevo Leon, University Hospital 'Dr. Jose Eleuterio Gonzalez' (Monterrey, Mexico) and the High Specialty Medical Unit (UMAE 25) of the Mexican Social Security Institute (IMSS) and the Century XXI National Medical Center, IMSS. This project was authorized by the Ethics Committee of the Autonomous University of Nuevo Leon Medical School and University Hospital (approval no. BI14-009) and by the National Committee of Bioethics of the IMSS (approval no. R-2012-785-075). All participants signed an informed consent letter.

The inclusion criteria included the following: Men and women aged >18 years with a diagnosis of CRC who wished to participate in the study and who signed the informed consent letter. As part of the study participants had to undergo CRC surgical removal. The exclusion criteria included the following: Patients with a family history of CRC who did not sign the informed consent letter, pregnant women, Karnofsky Scale <60. Clinical characteristics and sociodemographic data are shown in Table I. The quality of life of the participants was valued immediately before surgery and 24 months after starting treatment using the Karnofsky Scale (27).

Experimental strategy. Fresh sample fragments (>1.4 cm^2) were obtained from CRAC, normal tissue adjacent to the tumor (NAT; 3-7 cm distance from tumor) and normal colon from a donation of the historical collection of unidentifiable samples from the Department of Forensic Medicine of the University Hospital of the Autonomous University of Nuevo Leon. Normal colon tissues were preserved in RNA later stabilization solution at -20°C (Thermo Fischer Scientific, Inc.). Each sample was then divided into four fragments. A random fragment was used for chemosensitivity assays in a primary culture assay. A second fragment was fixed with Carnoy's solution, which contained ethanol, chloroform and glacial acetic acid (6:3:1 ratio), for 24 h at room temperature, embedded in

paraffin, cut into 4-μm thick sections and mounted on glass slides for hematoxylin and eosin stain (H&E) and immunofluorescence (IF) analysis. A third CRAC fragment was immersed in RNAlater (Thermo Fischer Scientific, Inc.) for 48 h at 4°C. RNA was isolated using RNAeasy mini kit (Qiagen GmbH). Finally, a fourth CRAC fragment was used to isolate CSCs by culture media.

Primary cultures (PCs). A piece of tissue (CRAC, NAT and normal colon) from each sample was washed with sterile phosphate-buffered saline (PBS; Sigma-Aldrich; Merck KGaA), immersed in 70% ethanol for 1 min (Sigma-Aldrich; Merck KGaA) and then cut into small pieces (<1 mm^3) and mixed with 100 UI/ml Collagenase Type I (Invitrogen; Thermo Fisher Scientific, Inc.) diluted in 4 ml RPMI culture medium (Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml gentamicin (Gibco; Thermo Fisher Scientific, Inc.) and 2.5 μg/ml amphotericin B (Invitrogen; Thermo Fisher Scientific, Inc.). Next, the preparations were incubated for 2 h in a water bath at 37°C with a magnetic stirrer (8 stirs/min) on Cimarec hot plates (Cimarec; Thermo Fisher Scientific, Inc.). The disaggregated cells were separated from debris by sifting each of the digested preparations through a 100 μM mesh cell strainer (BD Biosciences). Then the cells were washed three times with PBS followed by resuspension in 1 ml Iscove's Modified Dulbecco's Medium (IMDM; Gibco; Thermo Fisher Scientific, Inc.) with 100 μg gentamicin/ml, 2.5 μg amphotericin B/ml (Gibco; Thermo Fisher Scientific, Inc.) and supplement 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). Preparations were then analyzed using the trypan blue exclusion test for cell viability. Briefly, 0.4% trypan blue solution was added to cells at room temperature. Immediately after cells without the stain (viable cells) were counted using a hemocytometer.

Determination of anti-CRAC first-line drug cytotoxicity. The evaluation of cytotoxicity in response to a mixture of first-line drugs (5FU, OXA and leucovorin) was performed with the modified ATP-CRA assay (28). Briefly, viable cells were inoculated in triplicate (20,000/50 μl) from each piece of tissue, as well as from a cell line of colorectal adenocarcinoma Colo 320DM from ATCC, in a 96-well ultralow attachment plate (Costar; Corning, Inc.) with IMDM supplemented and 10% FBS. These cells were then divided into microplates; six wells for the negative control, three wells for treated cells, and three wells contained IMDM medium as a blank, without cells or drugs. As an internal control, 105 pg ATP diluted in 100 μl IMDM medium/well was added into three wells in each microplate. Next, the mixture of the chemotherapeutic agents were used (50 μl) at a final concentration: 10 μg 5FU/ml (Teva Pharmaceutical Industries, Ltd.), 2.9 μg OXA/ml (Asofarma de México) and 0.5 μg leucovorin/ml, which represent the maximal plasma concentration of each drug. After 48 h of incubation at 37°C and 5% CO$_2$, the cells were lysed with 100 μl lysis buffer/well, which was then included with the CellTiter-Glo kit (Promega Corporation). The ATP content was measured in each well with the luminometer Cytation 3 (BioTek Instruments, Inc.). Luminescence was equivalent to the ATP production by cells as described elsewhere (28). The percentage of cell death (PCD) was determined as the rate of ATP luminescence reduction in the treated cultures concerning

Table I. Clinical characteristics of patients (n=51).

Characteristics	Number of patients, n (%)
Sex	
Female	17 (33.0)
Male	34 (67.0)
Age, years	
30-49	8 (16.0)
50-59	17 (33.0)
60-69	15 (29.0)
>70	11 (22.0)
Location	
Rectum	18 (35.0)
Colon	33 (65.0)
Histopathological diagnosis	
Adenocarcinoma	51 (100.0)
Moderately differentiated	40 (78.5)
Poorly differentiated	4 (7.8)
Others	7 (13.7)
TNM	
T2N0M0	10 (19.6)
T2N2M0	5 (9.8)
T3N0M0	19 (37.3)
T3N1M0	9 (17.7)
T3N0M1	4 (7.8)
T4N0M0	3 (5.9)
T4N2M0	1 (1.9)

TNM, tumor, node, metastasis.

the untreated controls. Tissue with PCD ≥15.0±5.0 was classified as drug-sensitive and PCD ≤15.0 as drug-resistant. Cytotoxicity was evaluated as previously reported (28).

H&E and IF staining. A total of two histological sections were obtained from each fragment. One of these sections was stained with H&E to verify the orientation of each tissue. The other section was processed for IF to visualize *in situ* and calculate CD44 and CD24 percentage, using monoclonal primary antibodies CD44-PE-Cy7 (cat. no. ab46793) and CD24-FITC (cat. no. ab30350; Abcam). Each antibody was diluted with PBS 1:100 v/v. Next 100 μl diluted antibody was added by slide (50 μl from each diluted antibody) and incubated at 4˚C overnight. For each slide, 10 μl of mounting medium with DAPI Vectashield (Vector Laboratories, Inc.) was used to stain the nuclei of cells blue. Finally, all slides were stored at 4˚C in the dark until viewed with an epifluorescence microscope (Nikon Eclipse 50i; Nikon Corporation). Colo320DM was used as the positive control and as the negative control, histological sections of the brain (negative control tissue) and colon (without primary antibody) were used.

All preparations were observed with an epifluorescence microscope at a magnification of x400. From each of the histological sections, five microscope fields were chosen for image capture using a digital camera (Digital Sight DS-L2; Nikon Corporation). Microscope fields were chosen with the aid of the software, ENIS-Elements B12 v2.30 (Nikon Corporation). Finally, the results were reported as the mean ± standard deviation of CD44$^+$ and CD24$^+$ in the five microscope fields of all CRACs, NAT, normal colon and COLO 320DM. The percentage of CD44$^+$, CD24$^+$ and CD44$^+$/CD24$^+$ cells was estimated regarding the total number of cells in each histological section, by applying the following equation:

PPC (percentage of positive cells)=the number of positive cells/the total number of cells or nuclei observed by DAPI x100.

P<0.05 by Student's t-test was considered to indicate a statistically significant difference.

Double-labeled immunohistochemistry kit confirmed the expression level of CD44 and CD24 (HRP/Green & AP/Fast Red; Abcam) according to the manufacturer's protocol, using monoclonal primary antibodies anti-CD24 (clone ALB9; 1:100) or anti-CD44 (clone F10-44-2; 1:200) and anti-CD26 (clone: polyclonal; 1:200). A total of six sensitive and six resistant tissues were evaluated to confirm a significant difference between the two groups analyzed. Slides were scanned with an Aperio AT2 Digital Pathology Scanner (AT2 Model; Leica Microsystems, Inc.), evaluating the intensity of the signal using the Sketch and Calc program (iCalc Inc., 2018; https://www.sketchandcalc.com/).

RNA-total isolation from CRACs. A total of 34 fresh whole samples of CRACs were submerged in 1.5 ml RNAlater for 48 h at 4˚C. Afterwards, RNA was isolated using an RNAeasy mini kit and was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.), The RNA was kept at -80˚C until ready for use. Also, RNA was quantified with RiboGreen™ (cat. no. R11490; Thermo Fisher Scientific, Inc.) using the Qubit instrument and integrity was analyzed using the Bioanalyzer RNA 6000 NanoAssay Kit, Agilent 2100 Bioanalyzer system (Agilent Technologies, Inc.). The remaining RNA was stored at -80˚C until the samples were processed to construct the libraries.

Isolation and expansion of CSC. Isolation and expansion of CSC were performed as described by the modified method of Cammareri *et al* (29). Briefly, cells from disaggregated tissue CRAC or Colo320DM were seeded in 100 μl/well (5x10^4 cells suspended in 5 ml of medium) in 96-low adhesion plates and incubated at 37˚C with 5% CO_2. Cell growth every third day was observed using an inverted microscope (VE403; Velab). During this period, 50 μl of fresh Dulbecco's Modified Eagle Medium (DMEM) F12 culture medium enriched with 6 mg glucose/ml, 1 mg $NaHCO_3$/ml, 5 mM 4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 2 mM glutamine, 4 μg heparin/ml, 10 ng basic fibroblast growth factor (bFGF)/ml, 20 ng epidermal growth factor (EGF)/ml, 100 μg human transferrin/ml, 25 μg insulin/ml, 9.6 μg putrescin/ml, 30 nM sodium selenite and 20 nM progesterone was added every 3-5 days to each well [all the above reagents were purchased from Sigma-Aldrich (Merck KGaA)]. Cultures were incubated at 37˚C in a 5% CO_2 atmosphere for 15-20 days until typical CSC spheroids were observed.

Identification of CD44+ and CD24+ CSCs and differentiation of epithelial cells. CSC isolates (n=3 with 500,000 cell/ml) 100 μl were seeded in a slice coated w/poli-L-lysine, after fixation with Carnoy's solution for at least 48 h at 4°C. Next, quantification of CD24 and CD44 cell surface markers was performed by IF as previously mentioned. Then, five fields by color for each sample were counted by two independent observers. The graphs represented the mean obtained ± standard error. For differentiation of CSC to epithelial cells, 1x10^3 CSCs were incubated in each well of the four microchambers of a Nunc Lab-Tek II Chamber Slide (Nalge Nunc International; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO$_2$ atmosphere for 25-30 days, with RPMI 1640 supplemented gentamicin, amphotericin B and 2% bovine serum albumin (SAB; Sigma-Aldrich; Merck KGaA), culture medium was replenished every third or fourth day. Visualization of the morphology of the cells was performed with an inverted microscope (VE403; Velab). The concentration of FBS in the culture media was increased on day 3-4 to 5% and on day 9-10 to 10% and after day 15 to 15%. The differentiated cells were fixed with methanol-acetone (1:1 v/v) and stored at 4°C until their analysis with *in situ* IF. Subsequently, epithelial cells were characterized using primary monoclonal antibodies 1:200 in PBS (v/v) (Abcam), anti-CK-17 (cat. no. ab51056), anti-CK-20 (cat. no. ab109111), a mixture of monoclonal antibodies against a cocktail of CK (cat. no. ab115959) signals were detected using secondary polyclonal antibodies anti-IgG [1:500 in PBS (v/v) Alexa Fluor 488; cat. no. A32731; Invitrogen; Thermo Fisher Scientific, Inc.] and anti-EGFR (cat. no. ab32562) detected secondary antibodies polyclonal anti-IgG [1:500 PBS (v/v) Alexa Fluor 660; cat. no. A-21073; Invitrogen; Thermo Fisher Scientific, Inc.]. From three randomly selected fields, images were captured at x400 magnification. These images were merged using ImageJ version 1.52r (National Institutes of Health).

Characterization of Colo 320DM- and CRAC-CSC. From CSCs (2,000 cells/well), resistance to 5FU/OXA was determined by ATP-CRA after 48 h of exposure to the mixture (5FU and Oxa). Then the cells (~500,000) were washed twice with PBS and incubated with fresh CSC-medium without 5FU/OXA. From these cultures (CRACs- or -Colo320), one cell fraction was selected with magnetic beads by binding anti-CD44 monoclonal antibodies. Cells were passed through an LS Column and a MidiMACS Manual Separator (Miltenyi Biotec, Inc.). Next, the eluted cell suspension was centrifuged at 500 x g for 10 min at room temperature, discarding the supernatant and resuspending the cells in 500 μl RNAlater and left at 4°C for 48 h after isolating total RNA.

cDNA obtention and sequencing. RNA-seq libraries were constructed using 50-250 ng RNAs (RIN>7) with TruSeq Target RNA Expression System (Illumina, Inc.). The libraries were denatured as single-stranded DNA molecules, captured on Illumina flow cells, amplified *in situ* as clusters and finally sequenced for 150 cycles on an Illumina MiSeq Sequencer (Illumina, Inc.) according to the manufacturer's instructions. The cDNA library was constructed using the TruSeqRNA Access Library Prep system (Illumina, Inc.). Libraries with concentrations ≤2 nM were discarded. The size of cDNAs

was determined with a 2100 Bioanalyzer system (Agilent Technologies Deutschland GmbH). The concentration of cDNAs was determined with Pico green (Thermo Fisher Scientific, Inc.), following the instructions of the manufacturer. cDNA sequencing was performed with 10 pM of the libraries with 0.1% PhiX. Single-end sequencing of multiplexed cDNA libraries was carried out on an Illumina MiSeq sequencer for 150 cycles (Reagent kit v3; Illumina, Inc.).

Normalization of cDNA data. Data from four cDNA sequence-readings was obtained using the following equation: N=Total number of readings of each gene (TNRG)/Total number of readings per sample (TNRS) x Total number of readings of all sequenced cDNA (TNRAS). Where N denoted normalized data; TNRG, the total number of readings of each gene, TNRS, the total number of readings per sample and TNRAS, the total number of readings of all sequenced cDNA. Over 94% of reads were identified, accepting >Q30 (Q30=1 error each 1,000 bp).

Bioinformatics analysis. To visualize the differences in gene expression associated with resistance, heatmaps were developed using GraphPad Prism 6.0 program (GraphPad Software, Inc.). The number of normalized readings for each of the samples of sensitive, resistant and control (healthy) tissues were considered and plotted. Using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) program (https://string-db.org/), the STRING protein-protein interactions were evaluated to determine whether these genes were involved in any common metabolic pathways or functions.

KRT-18 analysis on CRAC. Based on the results, one gene, *KRT-18*, with overexpression of resistance of CRAC and CSC was chosen. To confirm the results, we analyzed co-expression of KRT-18 and CD44 by IF using another 12 different paraffin-embedded CRAC only from stages III or IV (six tissues classified as drug-sensitive and drug-resistant) and six healthy tissues.

Statistical analysis. Data for chemosensitivity of primary cultures of CRAC and healthy colon samples were performed in triplicate and analyzed using Bonferroni's correction following one way ANOVA, variance analysis and Student's t-test. The percentages of the markers CD44, CD24 and CD26 were obtained in duplicate from different sections of tissues of each patient in five fields and two independent observers made analysis using Kruskal-Wallis (ANOVA-means and ranges) and Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Statistical analyses were performed using the SPSS version 20 (IBM Corp.). Results of CRAC analysis showed a PCD ≥15±5% standard deviation and a percentage ≥5 of CD44$^+$ and CD24$^+$ cells were considered sensitive to 5FU/OXA. From this, a heat map was constructed to show the number of normalized readings using the GraphPad Prism 8 program (GraphPad Software, Inc.). The levels of gene expression by the CSCs from sensitive and resistant CRACs were then compared with Basespace Illumina software version 4.10 (Illumina, Inc.) and Euclidean distance (absolute difference between average normal expression or sensitive CRAC
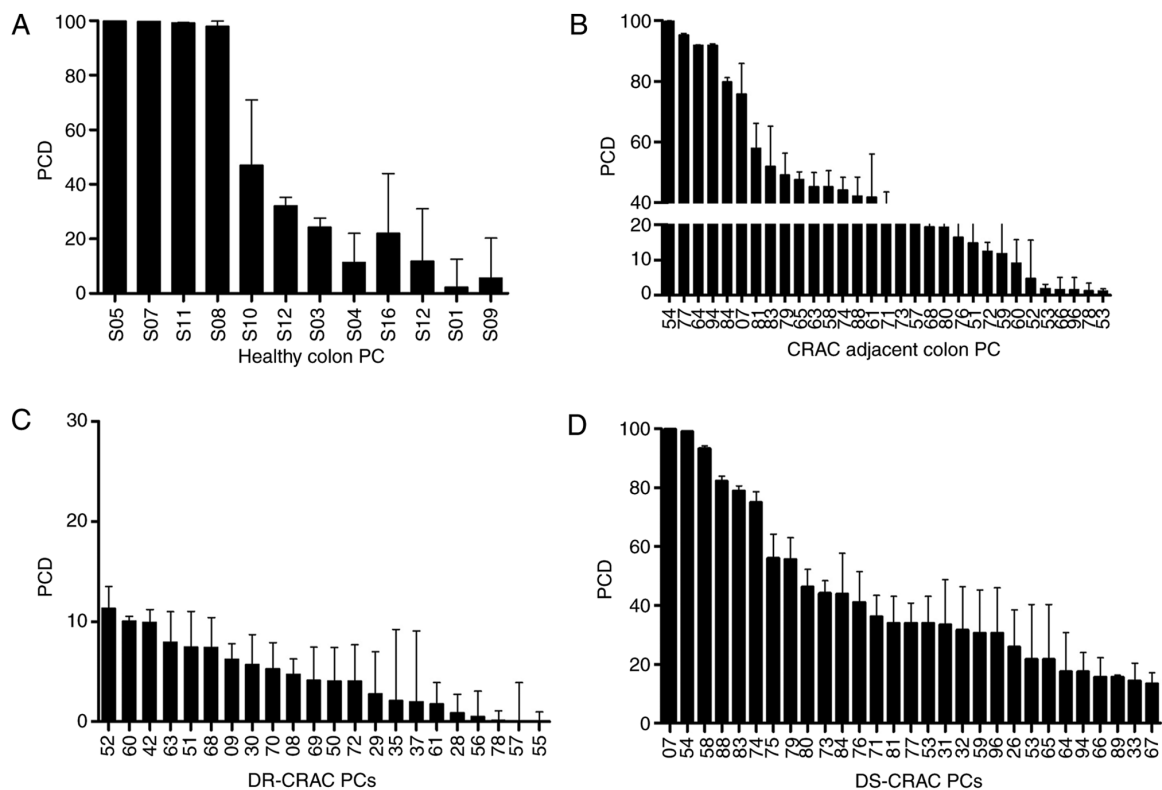
Figure 1. Cytotoxicity response to 5-fluorouracil and oxaliplatin in primary cultures. (A) Healthy colon tissue (n=12). (B) Adjacent tissue of CRC (n=31). (C) Resistant CRC primary tissue (n=22). (D) Sensitive CRC primary tissue (n=29). The black bars correspond to the mean ± standard deviation of the percentage of cell death response from each sample in triplicate. CRC, colorectal cancer; PCD, percentage of cell death; PC, primary cultures; CRAC, colorectal adenocarcinoma; DR, drug resistant; DS, drug sensitive.

and resistance CRAC). P<0.05 was considered to indicate a statistically significant difference. To identify which genes were relevant, genes found with overexpression in normal colon tissue and human mesenchymal stem cells isolated from adipose tissue (hMSC-AT) were discarded.

Finally, sets of data were analyzed by one-way ANOVA followed by the Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Variability of drug-sensitivity in CRACs-PCs. To determine the threshold to define the sensitivity of the tumors, some adjacent regions from tumor and healthy tissues were evaluated with the same experimental technique. All 12/12 samples from healthy tissue (100%) had PCD ≥15.0±5.0 and were classified as drug-sensitive, Fig. 1A and B show that 22/31 (71%) CRAC-adjacent colon samples were sensitive to treatment. Then the response to 5FU/Oxa on all primary cultures (PCs) was analyzed as seen in Fig. 1C and D. This demonstrated that CRAC 22/51 (43.1%) samples were drug resistant and 29/51 (56.9%) were sensitive. Also, 14 samples (44%) from the same person (CRAC and CRAC-adjacent colon) were resistant and 17 samples (56%) were sensitive. Colo 320 was a sensitive control (92.70±00.22).

CD24+ and CD44+ markers in CRACs. CRACs expressing CD24 and CD44 were then tested for sensitivity and resistance to 5FU and oxaliplatin. Results shown in Fig. 2A-D.

Fig. 2E-G show the mean ± standard deviation of CD44- and CD24-positive markers on sensitive and resistant CRACs. Briefly, the percentage of CD44+ cells was 4.80±2.84% and for CD24+ 3.85±1.48%. The mean ± standard deviation of CD44+/CD24+ cells was 2.70±2.075. By contrast, the positive percentage of CD44+ and CD24+ cells in the resistant tumor were 11.75±13.12 and 9.86±9.18%, respectively. There was a significant difference in CD44+ and CD24+ (individual markers) as sensitive and resistant tissue, as shown in Fig. 2E and F.

Quality and integrity RNA total CRACs. Total RNA (n=51) was then obtained from the CRACs cells (50-1,200 ng/μl) and the integrity analyzed. This resulted in 34 samples with a RIN >7, which were later used for the analysis of gene expression. In addition, 10 RNA samples (80-849 ng/μl) from healthy tissue had higher quality and integrity and were chosen for analysis (data not shown). Results were public and added to https://www.kaggle.com/elsangarzatrevio/qubit-and-bioanalyzer/version/1. None of the adjacent colon to CRAC was used for RNAseq.

Isolates and characteristics of CSCs. A total of three CSC cultures from CRACs of advanced cancer tissue and from cell line Colo320 were successful and were used as a control. CSC isolates were positive to CD44 and CD24. These results demonstrated an eight times higher expression of markers after culture, as shown in Fig. 3A-F. It was also found that differentiation of CSC to epithelial cells occurs only after
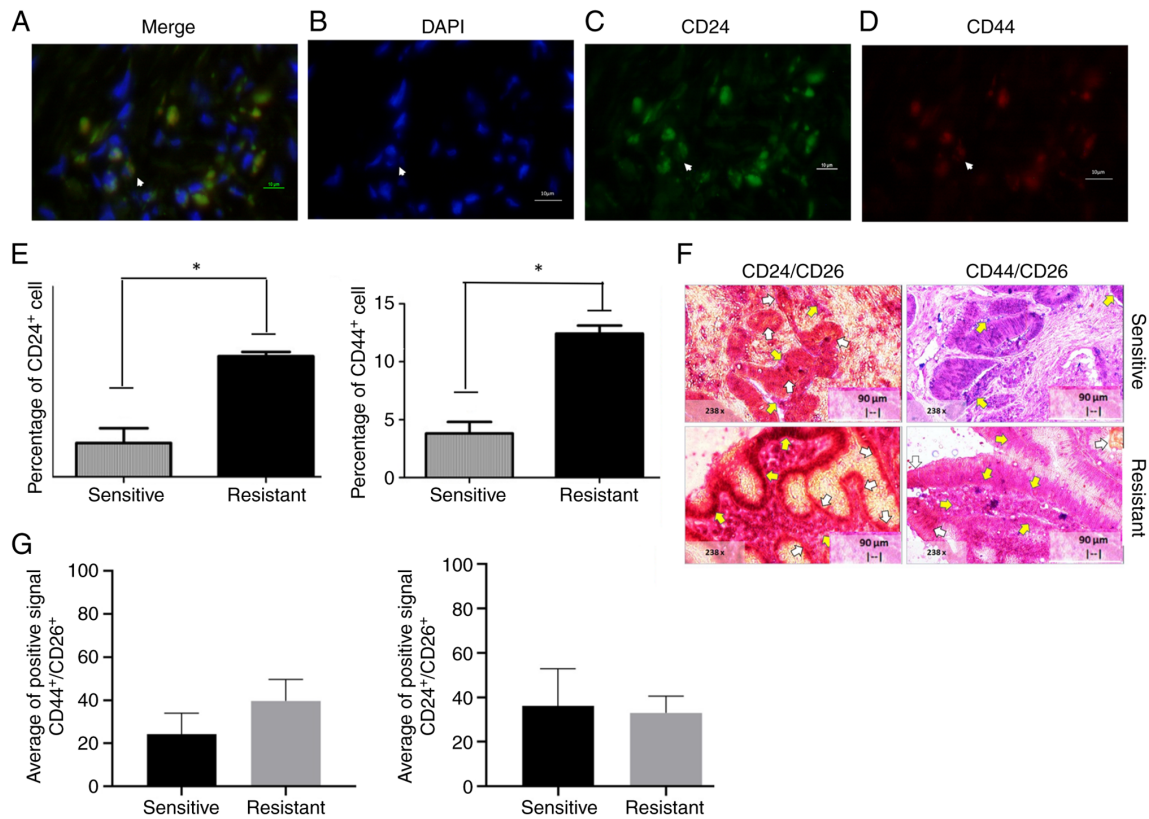
Figure 2. Percentage of CD44+ and CD24+ cells from tumor tissue of patients with CRAC classified as sensitive or resistant to 5-fluorouracil and oxaliplatin. (A-D) Expression CD44 and CD24 in resistant CRC tissue (magnification, x40). Arrows indicate positive signal. Representative images of immunostaining CD44 and CD24 (A) merge, (B) DAPI (blue), (C) CD24+ coupled to FITC (green signal) and (D) CD44+ coupled to PE-Cy7 (red signal). The nuclei of all the cells are stained with DAPI. (E) Percentage of CD24+ cells and percentage of CD44+ cells. (F) Representative images of double immunostaining CD44 and CD26 or CD24 and CD26; merge signal is purple (yellow arrow), CD44 or CD24 color green and CD26 signal red (white arrow). Scale bar, 90 μm. (G) Average expression of CD44/CD26 and CD24/CD26. In E and G the bars correspond to the mean ± standard deviation of the percentage of CRAC resistance. *P<0.05. CRAC, colorectal adenocarcinoma; CRC, colorectal cancer.

15 days in medium with serum. Notably it was observed that cells acquired the ability to adhere to plastic and express EGFR (Fig. 3G), CK17 (Fig. 3H) and CK20 (Fig. 3I) markers (<80%).

CSCs were further analyzed for chemoresistance by an ATP-CRA modified assay. It was also observed that cell lines from Colo320 maintained in medium enriched CSC, permitted reduced cytotoxic activity of the drugs assayed. Results showed a decrease (Fig. 4) from 88.43±7.17 to 72.36±4.78 (P<0.05). However, CSCs isolated from tumor tissue were resistant before and after they were in the medium to enrich CSCs. After eliminating the stimulating chemotherapeutic agents, tumor cells that proliferated were observed.

Genes overexpressed by CSCs from Colo320DM and CRACs. A total of 66 genes were selected that were associated with chemoresistance of 5FU and oxaliplatin-based in the project (ID 40581) created with *Homo sapiens* (human) genome assembly GRCh37 (hg19) from Genome Reference using the Illumina platform. Next Generation Sequencing (NGS) technologies were used to assess overall gene expression profiles. Pre-validated primers for the conserved region and specific targets were selected and are shown in Table II. Gene expression levels in healthy colon samples and CSC resistance to 5FU and oxaliplatin were compared. Protein-protein interactions were also analyzed using STRING, to ascertain if the overexpression protein belonged to the same pathway or metabolic route. A heatmap of the Colo 320 line was constructed before and after treating CSC in medium (Fig. 5A); the genes that were found highly overexpressed were: Excision Repair 1, Endonuclease Non-Catalytic Subunit (ERCC1), Poly-ADP-ribosyltransferase (PARP1), TGFβ, ATP binding cassette subfamily B member 1 (ABCB1) and MYC.

Resistant and sensitive tissues had the variability of the expression genes analyzed; however, greater expression in sensitive tissues of genes involved in cell growth and differentiation was observed. By contrast, in resistant tissues, the main overexpressed genes were SOD1, MYC, PARP1, HGD, BSTG1, PKM2, GSTM1, ALDH3B and ABCB1 (Fig. 5B).

The heatmap in Fig. 6A shows the expression levels of normal stem cells human-mesenchymal stem cells-isolate from adipose tissue and CSC isolates. As observed on the expression scale, the intensity of color allowed the observation of low expression levels in blue and high expression levels in red. Results of the CSC isolates from CRACS were different in each patient; however, overexpressed genes repeated with high frequency were ribonucleoside-diphosphate reductase large subunit (*RRM1*), *EGF*, *HGD* and *KRT-18* in colon CSC from patients with CRC (Fig. 6A). In addition, Fig. 6B represents relative expression levels of genes from CSC isolates and the tumor. It was found that *RRMI*, *EGF*, *HGD* and *KRT-18* genes were expressed in CSC tissue.
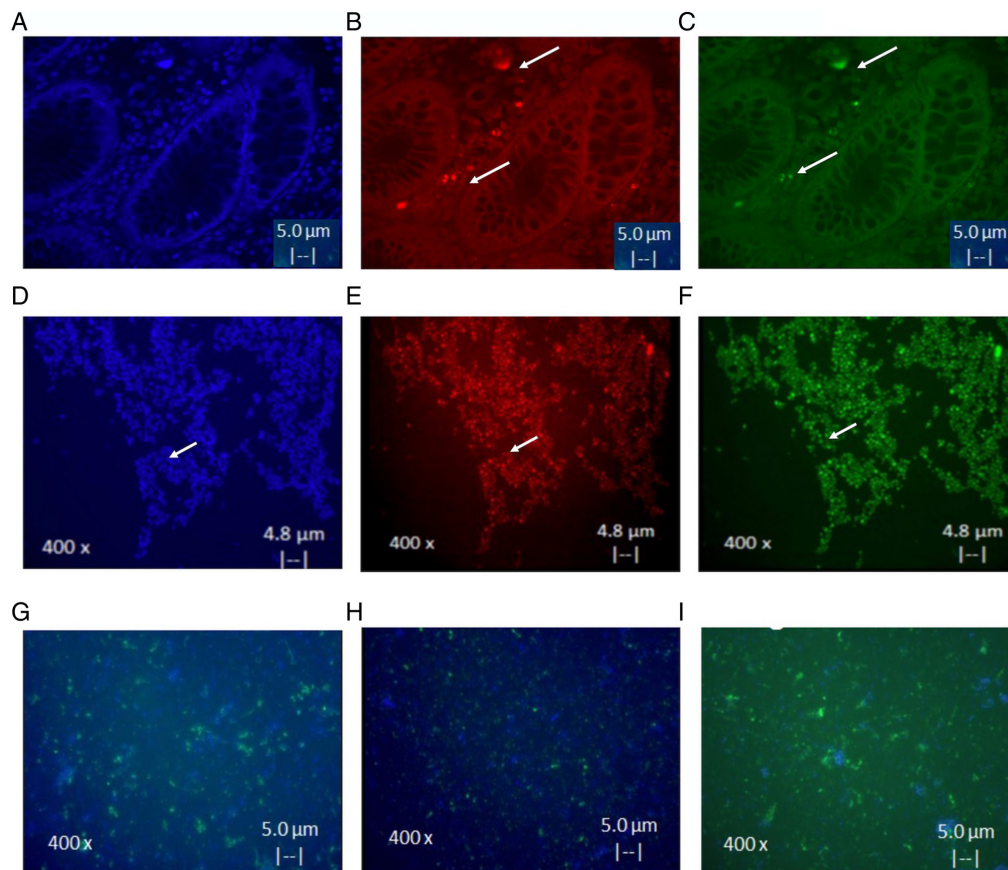
Figure 3. Characterization by immunofluorescence of CSC. (A) Representative images of cell nuclei stained with DAPI (blue) in CRAC. Immunostaining with (B) CD44+ marker coupled to PE-Cy7 (red signal) and (C) CD24+ marker coupled to FITC (green signal) in CRAC. Scale bar, 5.0 μm. Representative images of (D) cell nuclei with DAPI and immunostaining with (E) CD44+ marker coupled to PE-Cy7 (with red signal) and (F) CD24+ marker coupled to FITC (green signal) in one CSC isolated from CRAC tumors. Scale bar, 4.8 μm. Representative image of differentiated CSCs to epithelial cells with (G) EGFR+ marker, (H) CD17+ marker and (I) CK20+ marker. Scale bar, 5.0 μm. Arrows indicate cells with positive signals. These were observed with an epifluorescence microscope. CSC, cancer stem cells; CRAC, colorectal adenocarcinoma; EGFR, epidermal growth factor receptor.
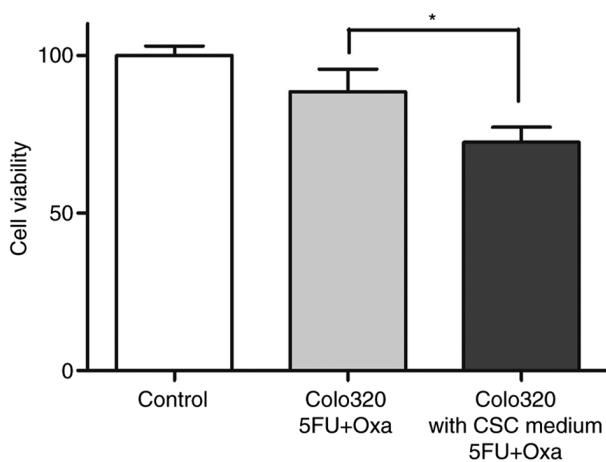

Figure 4. Cytotoxicity response to 5FU and Oxa on Colo320 cells. Bars correspond to the mean ± standard deviation of the percentage of chemosensitivity. *P<0.05. 5FU, 5-fluorouracil; Oxa, oxaliplatin; CSC, cancer stem cell.

Validation of resistant genes on CRAC. Following the results of the present and previous studies (30,31), *KRT-18* was selected as the key gene in the progression of CRAC to be validated by immunohistochemistry. Representative samples shown in Fig. 7A-F, demonstrated that co-expression of KRT-18 protein

and CD44 resistance was observed by IF in the 12 tissues from the patients with CRAC. No statistical difference was found between KRT-18 and CD44 markers in sensitive and resistant tissues. Calculated mean value for KRT-18 sensitive tissue showed positive for 2.27±1.9%, while resistant showed 3.18±4.03%, meanwhile CD44 sensitive showed 2.31±2.01%, whereas resistant showed 3.32±4.02% (data not shown).

Discussion

CRC is frequently associated with drug-resistance (32); in 90% of cases, this leads to cancer or tumor relapse. Drug resistance is situation frequently associated with tumor progression can induce mortality in patients (33). It has been further shown that the cells responsible for chemoresistance and metastasis are CSCs (19,21). Thus, CSCs could be the key to opportunely detecting chemoresistance and for finding a more adequate drug regimen. Certain markers are known to be in CSCs: Lgr5, CD44, CD24, CD26, CD29, CD166, CD326, CD133 and ALDH (28). However, these markers are not exclusive to CSCs and can also found in normal stem cells (34). One view is that these sets of markers are found in highly differentiable cells, although further research is warranted on the combined mechanisms for their expression, as this may lead to an improved understating of stemness and regulation (14).

Table II. Primers used for expression analysis.

Gene	Region	Forward primer (5'→3')	Reverse primer (5'→3')
VEPH	Chr3	CTTTGGAGCAAATTAAGATAATTAGCTCA	AACAATGACCAGGCAGTAGTTGAAATC
ERCC1	Chr19	GCTGGGCCAGAGCACCTGTGCCCTGTT	ACAACCTGCACCCAGACTACATCCAT
SLC4A4	Chr4	GATGGGCAGGAGATGGAGTGGAAGGAAA	AGAAAAAGTGGAACAGGGTGGGGAAA
HYAL1	Chr3	TTCTATGACACGACAAACCACTTTCTGC	TGGAGCACAGCCTGGGGGAGAGT
VNN1	Chr6	AGTAAACTGGATCCCTGTAATAATCG	CCAGTACAAGAAAGACTCAGCTGCCT
ANKRD44	Chr2	TGCTCATCCATAAAACTGAAGATGTGA	ACCCTCTTCATGTGGCCGCATTTCT
PROM2	Chr2	CCTCTCGGTGGTGCAGCTCAATCCTTT	GTTGGTAAAGGCCCTACTGAATGAGCT
DNER	Chr2	AGAAAGTTGTAGAAATGAAATGGGATCAAG	GGGAATGCCAGTTCTAACAGCTC
BTG1	Chr12	TCCTGCCGCCTCCTGTCTCGAAATAA	TCTAAAGAAAGAAAGACAAAGTAGTCGTC
CTPS2	ChrX	TCGATGCTGGCACTTTTTCACCTT	GTCTTAAATGATGGTGGAGAAGTTGAT
ITPA	Chr20	TCTGGAGAAGTTAAAGCCTGAAGGTC	GCCGGGTTCGAGGACAAGTCAGCCTAT
ENTPD5	Chr14	GGTGCTGTTAGGTAGGACTTGTATCCA	AAAGGAACCAAGGAGAAAATTCAGA
MLH1	Chr3	ATCTGAGGAGTCGACCCTCTCAG	CCAAACTCCTGGAAGTGGACTGT
PARP1	Chr1	TGGTGTGAATGACACCTCTCTACT	TATTGCTCAGGTAAATCTGAAGTATCTGC
UCK1	Chr9	CCTGATAGGGGTGAGCGGCGGCACT	TGTGTGAGAAGATCATGGAGTTGCTG
DPYD	Chr1	TAAATCATTCATCACAAGTATTGCAAACA	CTGCTAAGATGATATTTTCTGACAACCCAC
TYMS	Chr18	TATTTACCTGAATCACATCGAGCCACTG	CCAGACCTTTCCCAAAGCTCAGGATT
IL-23R	Chr1	TGGAATTATGTGCTTCAAACAGGTTGA	GGAAACAGTCTTTTCCTGCTTCCAGA
RNF180	Chr5	GTTTTATGGAGTATCTTGAGAATCAAGTG	ATGATTCAGTTGATGCTCAAAATATTTGTC
C70rf44	Chr7	ATTTTCAAGAGAGTTGTGCTATGATGTGG	GCAGGAAGCAGGCGGTCAATGCCTCT
GABRR1	Chr6	ATTTCAGCATGAGGCCTGGCTTT	TGTGGATGTGCAGGTGGAGAGTTT
CYP2C18	Chr10	TTCCAGTGGCTGAAAAAGTTAACAAAGG	ATGGAAAGAGATGGAAGGAGATCCG
ALDH3B2	Chr11	GACGTGCTGGCCCAGGACCTGCATA	TCTGAGCTCATCCTTTGCCAGAA
HGD	Chr3	ACACATAGAGGAGAGAGAAAATGGCTG	ACATTTCTGGATTTGGGAATGAGTGTTC
CLEC4E	Chr12	ATTCATCTAAATCATCTGAAACACAATGC	GAGAGGATGCTTCTCTTCCCAAATGT
ERAP2	Chr5	TTATAAAAGCACATACAGAACTCTTGGTGG	TGAGCCAACCCAGGCACGCATG
MTHFR	Chr1	CGAGCGTTCTGAGTCACCCGGGACT	CCCAGCCATGGTGAACGAAGCCA
GSTP1	Chr11	CAGGGAGGCAAGACCTTCATTGT	TACAACCTGCTGGACTTGCTGCTGAT
GSTT1	Chr22	TGAAGGACGGGGACTTCACCTTGA	CTACCTGACGCGCAAATATAAGGTC
GSTM1	Chr1	CAGCTGGGCATGATCTGCTACAAT	CAAAGTACTTGGAGGAACTCCCTGAA
MMRN1	Chr4	TACCAAAAATCAAATTTCGAAACAACTAG	TGTACATACCAGGTTATCTCCCACAGTG
CINP	Chr14	AGGAACTGCAGGCCACCTTGGAT	CCAAAATACAGGTGAAAATGGAAAGCTG
BRAF	Chr7	GGCCTCTTCGGCTGCGGACCCTGCCATT	TGGAATATCAAACAAATGATTAAGTTGACA
KRAS	Chr12	AGCGGCTCCCAGGTGCGGGAGAGA	GAATATAAACTTGTGGTAGTTGGAGCT
BNIP3	Chr10	TCTCTCATTTGCTGGCCATCGGATT	GATCTATATTGGAAGGCGTCTGACAACC
PSAP	Chr10	CTTCCTCCTGGCCAGCCTCCTG	TCCTTGGACTGAAAGAATGCACCAG
APC	Chr5	CTGGACAGATTGATTTATTAGAGCGTC	GCTTAACTTAGATAGCAGTAATTTCCCTGG
ABCG2	Chr4	AATGCAACAGGAAACAATCCTTGTAAC	TGTACTGGCGAAGAATATTTGGTAAAGC
ABCB1	Chr7	TGAAGCCACGTCAGCTCTGGATACAGAAA	CCCTGGACAAAGCCAGAGAAGG
NFKB	Chr4	ACTGCTGGACCCAAGGACATGGT	AACCTGGGTATACTTCATGTGACAAAG
AKT1	Chr14	GTGAGGCTCCCCTCAACAACTTCTCT	GTGCCAGCTGATGAAGACGGAG
TGFB1	Chr19	TACATTTGGAGCCTGGACACGCAGTA	ACAACCAGCATAACCCGGGCGC
TERT	Chr5	ACAAGCTGTTTGCGGGGATTCG	CTGCTCCTGCGTTTGGTGGATGATTT
BST1	Chr4	GCGCACTGCTGAGTCCCGAGCA	CAAGAACTGCACAGCCATCTGGGAA
STK17A	Chr7	ACGGCTACAGCCTGTGCCCGGG	CAGTGGTGAGAAAATGTATAAAGAAAGAT
NOTCH1	Chr9	AGAACGGGGCTAACAAAGATATGCAG	AGGAGACACCCCTGTTTCTGGC
MEG3	Chr14	GGGCGCCCACGAGAGGATCCCTCA	GGTCTCTCCTCAGGGATGACATCAT
TP53	Chr17	ACCATCATCACACTGGAAGACTCCA	AATCTACTGGGACGGAACAGCTTTG
PKM2	Chr15	TGATGGGCTTATTTCTCTCCAGGTGA	TGCCGACTTCCTGGTGACGGAGGTGGAAAA
WNT1	Chr12	GGCCCCACCTCTTCGGCAAGATCGTCAA	CGAGAAACGGCGTTTATCTTCGCTAT
RRM1	Chr11	ATGCACTTCTACGGCTGGAAGCA	GGTTTGAAGACTGGGATGTATTATTTAAG
KRT18	Chr12	CTTCAAGATCATCGAGGACCTGAGG	AATGCCCGCATCGTTCTGCAGAT

Table II. Continued.

Gene	Region	Forward primer (5'→3')	Reverse primer (5'→3')
EGF	Chr4	GCTGGTGAGGATGGCCAGGCAGCAGAT	AATGCAACCAACTTCATGGAGGCA
HIF1A	Chr14	TATTTGCGTGTGAGGAAACTTCTGGA	ATATTGAAGATGACATGAAAGCACAGATG
REPS1	Chr6	CGCAGCTGCCGAACGACGTGGTCCTA	ATGGAGCTTTGTGGTGCAACAAGA
MYC	Chr8	TGAGGAGACACCGCCCACCACCA	AGGAACAAGAAGATGAGGAAGAAATCG
CDK14	Chr7	AGCCCGGTTACTCTGCCTTCGT	TGTGTCACAAAGATGTCTACACGGAAC
EGFR	Chr7	CTGCCCGGCGAGTCGGGCTCTGGAGGAAAA	CAAGCTCACGCAGTTGGGCACTTTT
BCL2	Chr18	TTGACAGAGGATCATGCTGTACT	CAGAGGAAGTAGACTGATATTAACAATACT
SOD1	Chr21	ACCATTGCATCATTGGCCGCACA	CAGATGACTTGGGCAAAGGTGGAAAT
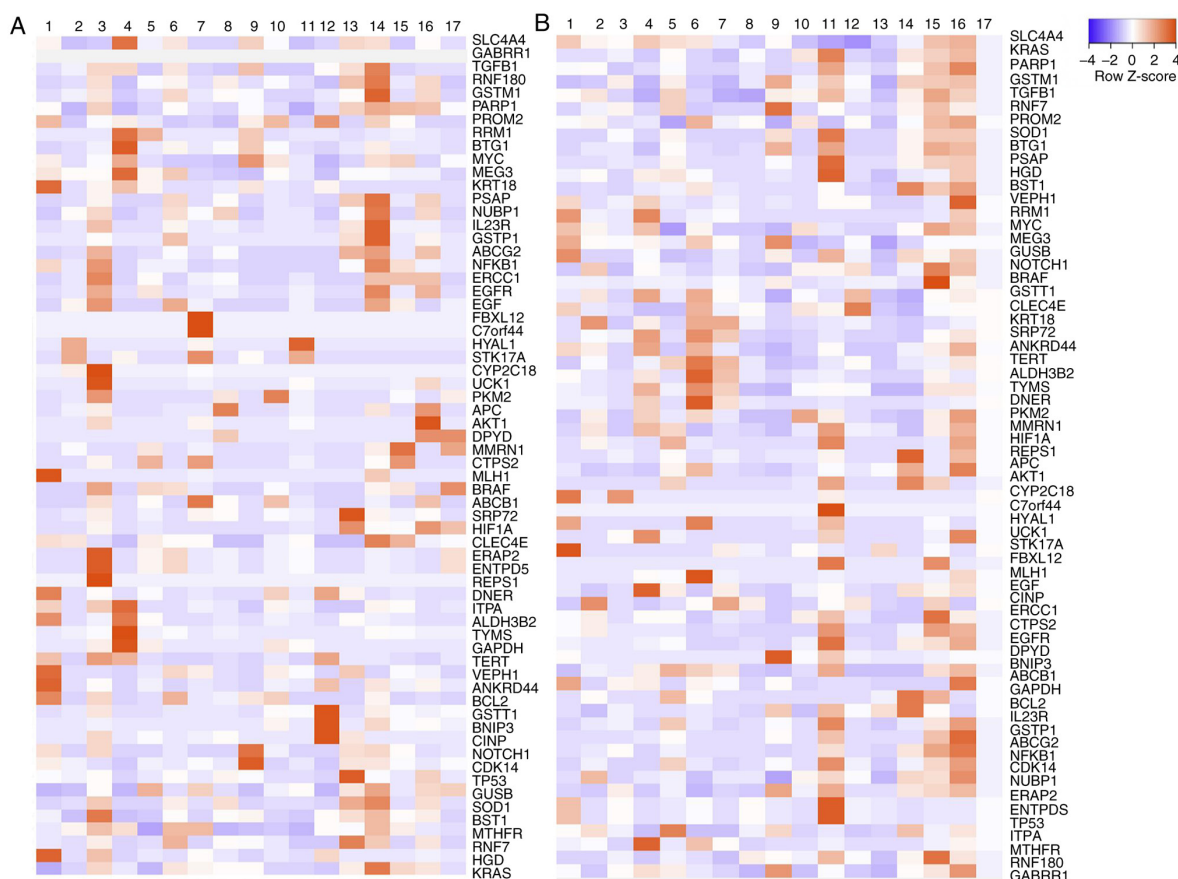
Figure 5. Heatmap of genes upregulated in patients with CRAC. Heatmap of patients with (A) drug sensitive and (B) drug resistant CRAC (red represents upregulation and blue color downregulation). CRAC, colorectal adenocarcinoma.

The present study identified *KRT-18* as a gene in CSCs of CRACs that could be a promising biomarker for 5FU/OXA resistance. The population analyzed corresponded mostly to adult men (49% <60 years) with advanced stages of CRAC, which is consistent with the fact that 91% of cases are diagnosed in individuals ≥50 years of age. Although the average age is similar to that reported, there are cases in individuals <50 years (15,33).

The present study classified all the primary cultures of CRACs as sensitive and resistant to 5FU/OXA. The condition of 5FU/OXA-sensitive or resistance was defined when the PCD was >15±5% as primary cultures of healthy colon are known to have a PCD >15%. This criterion agrees with that reported by Kwon *et al* (35) in 2016. The percentage of CRAC primary cultures resistant to 5FU/OXA was significantly (18%) higher than those sensitive to 5FU/OXA. It was notable that a subpopulation of primary cultures (23%) of the colon adjacent to CRACs were resistant to 5FU/OXA. This suggested that malignant cells of CRAC can induce malignancy in healthy tissue, as happens in other types of cancer (36), such as breast (37), melanoma (38) and prostate (39). The fact that CSCs and fibroblasts located in the tumor supports this, and the adjacent colon export cytokines, chemokines and miRNAs, which can cause normal cells to become malignant (40).

Typically, a high percentage of primary cultures are resistant to first-line antineoplastic agents, even before chemotherapy (41).
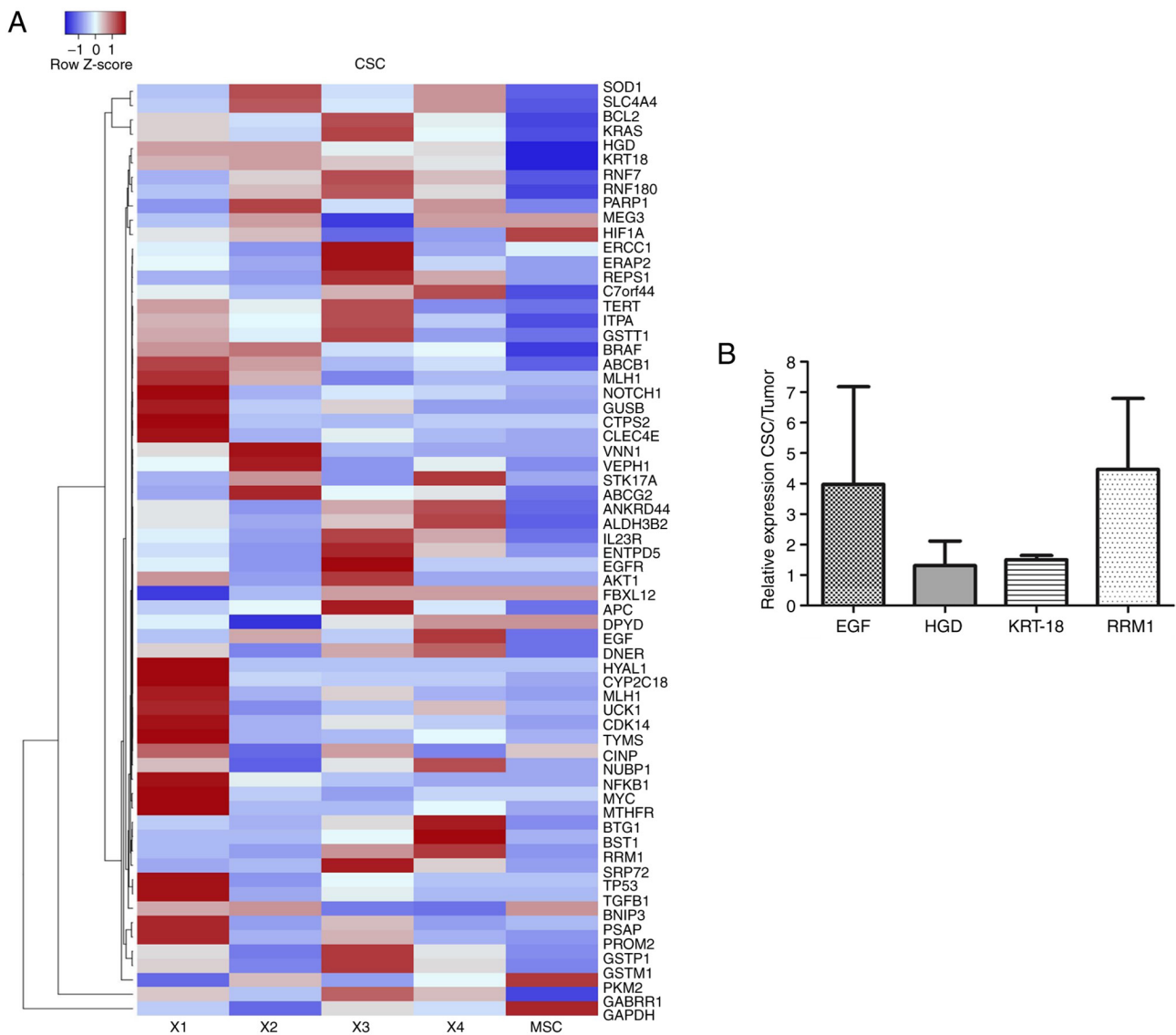
Figure 6. Genes upregulated in colon CSCs. (A) Heatmap of genes upregulated in colon CSCs (red represents upregulation and blue color downregulation). (B) Relative expression levels of genes from CSC isolates and the tumor. CSCs, cancer stem cells; KRT-18, keratin 18; HGD, homogentisate 1,2-dioxygenase; EGF, epidermal growth factor; RRM1, ribonucleoside-diphosphate reductase large subunit.

In most CRC cases, the primary tumor is completely resected; therefore, chemoresistance does not represent a problem in the first stage of cancer (29). However, in some patients, tumor cells remain in the gut of patients who were treated with a first-line drug, such as 5FU and OXA (42). In the present study, the Colo 320DM cell line was shown to be sensitive to all the drug combinations evaluated, which made it useful as an internal control each time a PC-CRC was analyzed (36,43).

CD44 and CD24 are putative markers to isolate CSCs from solid tumors activating and modulating several cell signaling networks, such as Wnt, NF-κB, Notch, Hedgehog, JAK-STAT, PI3K/AKT/mTOR and TGF/SMAD, that serve an important role in mediating tumorigenic properties of tumor cells leading to tumor progression, migration and associated with resistance to antineoplastic drugs; the present study found an increase of positive CD44 and CD24 cells in resistant CRAC tissue as previously reported (15,40).

Adapting and using a protocol reported by Cammareri *et al* (29) allowed us to isolate CD44+ and CD24+

CSC from primary cultures of CRC (29). The CSC isolate was successful in 7.8% of processed samples, as previously reported (13). This was especially true in the cases of patients with advanced cancer who had not received previous treatment with chemotherapy or radiotherapy, but were resistant to 5FU and oxaliplatin (higher percentage of CD44 and CD24 markers). Although it seemed low, it is similar to a previous study (13).

Previous results have also reported that colon CSCs were able to express LGR5 even after stimulation with irinotecan (42). In the present study, the selection of the resistant population was made by exposing the CSC to stimulation by chemotherapy and subsequent selection with anti-CD44 magnetic beads after 72 h (8). This allowed for the selection of cells resistant to 5FU and oxaliplatin; however, the results are limited by 5FU and oxaliplatin, therefore further analysis against other chemotherapeutic agents is required to understand all resistance profiles.

There was a substantial variation of certain genes observed in the results of NGS between the tissues with CRAC. Those
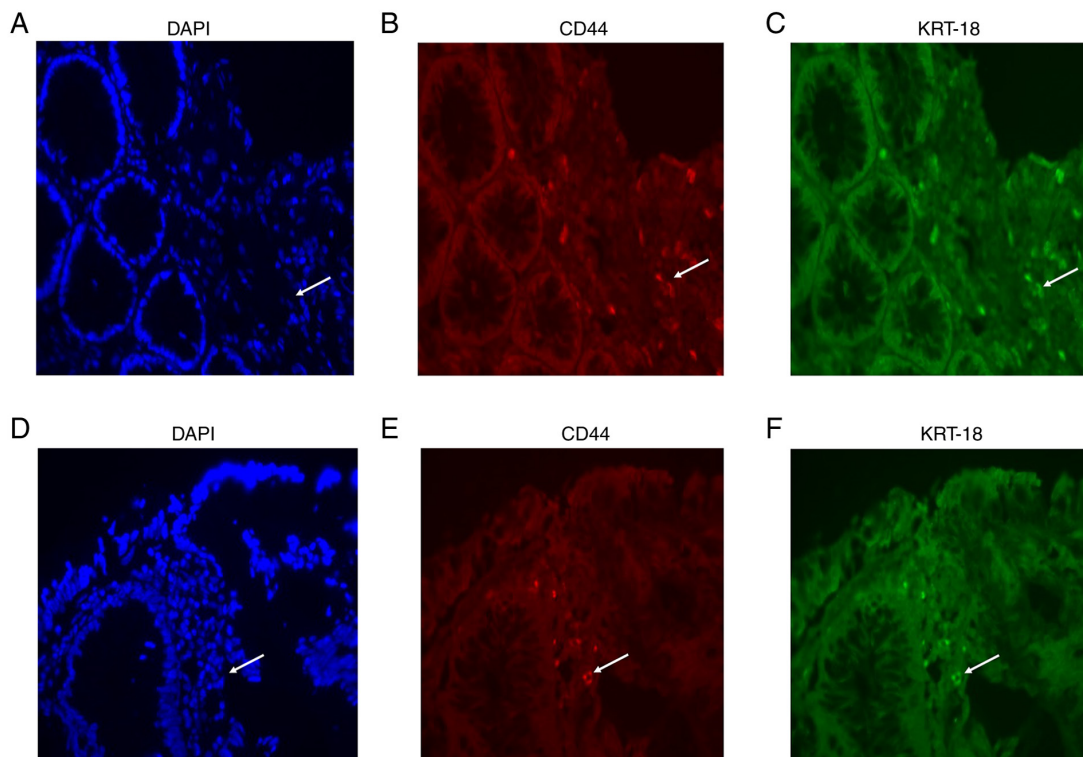
Figure 7. CD44 and KRT-18 expression in CRC tissue. (A) Representative image of resistant tumor tissue observed stained with DAPI (nuclei of the cells are blue). Immunostaining of (B) CD44⁺ marker coupled to PE-Cy7 (red signal) and (C) KRT-18⁺ marker coupled to FITC (green signal) on a resistant tumor. Representative image of sensitive tumor tissue (D) stained with DAPI (nuclei of the cells are blue) and immunostaining of (E) CD44⁺ marker coupled to PE-Cy7 (red signal) and (F) KRT-18⁺ marker coupled to FITC (green signal) on a sensitive tumor. Magnification, x40. Arrows indicate positive cells. CRC, colorectal cancer; KRT-18, keratin 18.

that were classified as sensitive presented a higher expression of genes involved in cell proliferation (*BTG1*, *MYC*, *MEG3*, *IL-23R*, *NFKB*, *ERRC1*) and detoxifying enzymes [Glutathione S-transferase μ 1 (*GSTM1*) and ATP-binding cassette superfamily G member 2]. On the other hand, genes involved in oxidative damage-apoptosis (*SOD1*, *MYC*, *PARP1*), metabolism alteration (*HGD*, *BSTG1*, *PKM2*) and detoxifying enzymes (*GSTM1*, *ALDH3B* and *ABCB1*) were observed with greater frequency in resistant tissues. Also in the results only advanced tumors with higher levels of CD44 and CD24 overexpressed TGFβ (30) and Hypoxia Factor Inducible 1 α (44), which are signals that induce expression of growth factors, such as fibroblast specific protein, smooth muscle actin α, vascular endothelial growth factor and pro-inflammatory cytokines (IL-6, IL-23 and/or IL-1β), which in turn participate and activate other pathways (WNT, Hedgehog and NOTCH) to promote EMT (8,13,21).

Subsequently, the expression of genes associated with resistance in the CSC isolates and the MSC-ADTs was compared. It was found that the genes with the highest expression levels were *HGD*, *KRT-18*, *RNF180*, *EGF* and *RRM1*. Notably, *HGD*-related genes in the tyrosine catabolic pathways have not been linked to changes in DNA, however, a dysregulated process exists in some types of cancer (45-47). A recent report has shown that tyrosine catabolic genes or microRNAs (miR-539 and miR-661) could be used as a prognostic biomarker for hepatocellular carcinoma (48). The RNF180 gene is a E3 ubiquitin ligase implicated in the ubiquitin-proteasome pathway, which acts as a potential tumor suppressor, exhibiting

a critical role in the suppression of cell proliferation and induction of apoptosis (49,50). In the present study, the *RNF180* gene was significantly overexpressed; a previous study in GC cells suggested that RNF180 has regulatory activity on STAT3 and pSTAT3 (49). In addition, EGF signaling is necessary for the maintenance of colon CSC and the upregulation in the isolates can be due to medium supplementation with EGF (51). *KRT-18* gene belongs to a family of intermediate filament genes that can affect carcinogenesis through various signaling pathways, including PI3K/AKT, WNT and signal-regulated ERK and MAPK (52). *KRT-18* is overexpressed in most types of human tumor and associates with clinical stage, tumor stage and metastasis stage in patients with esophageal cancer (52). In gastric cancer, high KRT-18 expression has been suggested to be associated with positive lymph nodes, advanced clinical stage and chemoresistance (30). However, the clinical significance and biological function of *KRT-18* is seldomly reported in CRC. A recent study reported that high KRT-18 expression can promote viability, migration and invasion of tumor cells (30). In colonic epithelial cells, Lähdeniemi *et al* (44) found that *KRT-18* interacts with Notch1 and regulates Notch1 signaling activity, which promotes CSC.

Recently, KRT-18 was proposed in gastric cancer as a CSC marker by proteomic results and was found as a marker in circulating tumor cells (CTC) in metastatic colorectal cancer (30). In the results of the present study, KRT-18 was overexpressed in CSCs and at the protein level co-expression with CD44 markers in CRC-resistant tissue was observed. In addition, Virag *et al* (53) reported that the upregulation of *PTPRO*,

KRT-18, *NDRG1*, *AVEN* and *ID1* in association with CSC-CRC chemoresistance to oxaliplatin and Francipane *et al* (54) reported that the upregulation of PTEN, MMAC1 and TEP1 is associated with CSC-CRC. Finally, the results of the present study demonstrated that resistant CRAC tissues in patients with a higher percentage of CSC were associated with resistance to 5FU and oxaliplatin with overexpression of *KRT-18*; a gene that could be used to predict sensitivity to conventional drugs in patients with CRAC. Therefore, further studies are warranted to explore the molecular mechanism of *KRT-18* in tumorigenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The sequencing datasets generated and/or analyzed during the current study are available in the Kaggle, Inc., repository, https://www.kaggle.com/elsangarzatrevio/rna-seq-crc?select=Tejidos+tumorales+y+sanos+normalizados+con+GADPH.csv.

Authors' contributions

ENGT contributed to designing and conceiving the experiments, and analyzing and discussion of the results. HGMR contributed to the standardization and design of the experiments, and wrote, analyzed and corrected the manuscript. PDG obtained informed consent, conducted sampling and evaluated cytotoxic assays. OSC helped in the CSC cultures, characterization and immunohistochemistry protocol. ROL led the experimental design and analysis of the results obtained in sequencing. ASD helped in the standardization of immunohistochemistry and with the software of the fluorescence microscope. VMT performed some of the bioinformatics analyses. GRPR performed statistical analysis.

AGQR contributed to data analysis and designed some of the figures. JFIC performed data analysis and interpretation, and edited and critically revised the manuscript. SLSF designed the experiments, analyzed literature and discussed and managed the approval to submit the manuscript. ENGT and PDG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This project was authorized by the Ethics Committee of the UANL Medical School and University Hospital (approval no. BI14-009; Monterrey, Mexico) and by the National Committee of Bioethics of the IMSS (approval no. R-2012-785-075). All participants signed an informed consent letter. In the letter, participants authorized us to analyze a sample of their intestinal cancer tumors and to use their sociodemographic and clinical data for the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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