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SNAI1-expressing fibroblasts and derived-extracellular matrix as mediators of drug resistance in colorectal cancer patients

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ABSTRACT

Resistance to antitumor treatments is one of the most important problems faced by clinicians in the management of colorectal cancer (CRC) patients. Cancer-Associated Fibroblasts (CAFs) are the main producers and remodelers of the extracellular matrix (ECM), which is directly involved in drug resistance mechanisms. Primary Normal Fibroblasts (NFs) and CAFs and cell lines (fibroblasts and tumor cells), were used to generate ECM and to identify its role in the oxaliplatin and cetuximab chemoresistance processes of CRC cells mediated by SNAI1-expressing fibroblasts. Matrices generated by Snai1 KO MEFs (Knockout Mouse Embryonic Fibroblasts) confer less resistance on oxaliplatin and cetuximab than wild-type MEF-derived matrices. Similarly, matrices derived from CAFs cause greater survival of colorectal cancer cells than NF-derived matrices, in a similar way to Snai1 expression levels. In addition, Snail1 expression in fibroblasts regulates drug resistance and metabolism gene expression in tumor cells mediated by ECM. Finally, a series of 531 patients (TCGA) with CRC was used to assess the role of SNAI1 expression in patients' prognosis indicating an association between tumor SNAI1 expression and overall survival in colon cancer patients but not in rectal cancer patients. SNAI1 expression in CRC cancer patients, together with in vitro experimentation, suggests the possible use of SNAI1 expression in tumor-associated fibroblasts as a predictive biomarker of response to oxaliplatin and cetuximab treatments in patients with CRC.

1. Introduction

Cancer development and progression depend not only on tumor cells, but also on the local tumor microenvironment (TME) where the malignant cells emerge. It is now assumed that tumor and surrounding stroma communicate in a paracrine way, which modifies the stroma and contributes to the malignant behavior of cancer by promoting tumor growth, angiogenesis and metastasis (Pietras and Östman, 2010). The TME comprises cellular and non-cellular components. The cellular component includes fibroblasts, endothelial cells, immune cells, mesenchymal cells, etc., while the non-cellular component includes mainly the extracellular matrix (ECM), but also growth factors and extracellular vesicles. Fibroblasts are usually the most abundant cells within the TME (Fiori et al., 2019; LeBleu and Kalluri, 2018). Within the TME, fibroblasts can be activated and altered by crosstalk with cancer cells, which transforms them into what are known as cancer-associated

Abbreviations: CRC, colorectal cancer; ECM, extracellular matrix; CAF, cancer-associated fibroblasts; NF, normal fibroblasts; TME, tumor microenvironment; EMT, epithelial-mesenchymal transition; MMP, metalloproteinase; WT, wild type; KO, knock out.

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fibroblasts (CAFs) (Xing et al., 2010; Cirri and Chiarugi, 2011). Unlike normal fibroblasts (NFs), CAFs express activation markers, usually including α-SMA, FSP-1 and PDGFR, and abnormally activated signaling pathways such as TGF-β1/SMAD (Yang et al., 2019). CAFs are indeed a heterogeneous population of cells that lack a specific surface marker. This can be explained by the diverse cell origins of fibroblasts, which can be derived from endothelial cells, adipocytes, stellate cells or immune cells, among others (Fiori et al., 2019). The several origins of CAFs could explain why they have been reported to have both tumorogenic and antitumorogenic effects (Sahai et al., 2020; Kalluri, 2016; Chen and Song, 2019).

The ECM comprises a network of proteins, glycoproteins, proteoglycans and polysaccharides that provides the scaffolding for cells in the TME (Nissen et al., 2019). Cell adhesion to ECM components is mediated mainly by integrin transmembrane receptors. Fibroblasts are the main producers of ECM components, playing an important role in turnover of collagen, the most abundant component in the ECM (Nissen et al., 2019; Levental et al., 2009). New structures forming support tumor cell migration and also serve as the scaffolding for new vessel formation (Fullár et al., 2015). The interaction between ECM, stromal cells and tumor cells, as well as growth factors and vesicles, plays a pivotal role in tumor behavior. ECM-cell interactions can activate cell mechanisms and pathways for cell-cycle regulation, migration, survival or gene expression. Collagen crosslinking and ECM stiffening increase integrin expression and, thus, cell-ECM adhesion and tumor progression (Levental et al., 2009). The ECM can also act as a growth factor reservoir, modulating its bioavailability for tumor cells and stimulating CAFs within the TME (Hynes, 2009). Importantly, the ECM's role also involves treatment resistance in cancer patients. It can hinder chemotherapy effects by acting as a physical barrier, impeding drug access to the tumor (Eke and Cordes, 2015a; Kesh et al., 2020; Leask, 2020). In addition, CAF-ECM interactions, mediated by integrins or FAK adhesion complexes, are known to have an impact on some tumors in response to chemotherapy (Kalluri, 2016; Eke and Cordes, 2015a).

SNAIL is a transcriptional factor with C-terminal zinc finger domain, encoded by the *SNAI1* gene (Baulida et al., 2019). SNAIL has a crucial role in epithelial-mesenchymal transition (EMT), a differentiation process by which the epithelial cell loses the epithelial phenotype to gain mesenchymal characteristics. In cancer, epithelial malignant cells acquire migration capacity due to EMT action (Batlle et al., 2000). Under normal conditions, adult fibroblasts do not express SNAIL, but it is expressed in activated fibroblasts in pathological situations (e.g. fibrosis or cancer) (Stanisavljevic et al., 2015). CAFs expressing SNAIL are related to increased deposition of fibers, such as fibronectin and collagen, and ECM remodeling (Rowe et al., 2009; Stanisavljevic et al., 2011). Accordingly, in previous studies, our group proposed SNAIL as a potential CAF activation marker (Herrera et al., 2014), also reporting its role in ECM remodeling and demonstrating that SNAIL-expressing CAFs increase ECM deposition and fiber alignment (Herrera et al., 2018). The anisotropic disposition of these fibers enable tumor cell migration and endothelial cell-mediated angiogenesis in the tumor microenvironment (Stanisavljevic et al., 2015; Herrera et al., 2018).

Colorectal cancer (CRC) is the second cause of cancer-related death (Bray et al., 2018). Adjuvant chemotherapy in CRC patients has been clearly shown to benefit stage III patients, increasing 5-year disease-free survival from 49.0% to 63.6%. Oxaliplatin plus fluoropyrimidine treatment for 6 months is the standard adjuvant therapy for these patients with stage III CRC (Böckelman et al., 2015). Otherwise, in metastatic CRC patients, with KRAS/NRAS/BRAF wild-type tumors, cetuximab (a monoclonal antibody to the epithelial growth factor receptor) demonstrated greater efficacy than chemotherapy alone (Biller and Schrag, 2021). In this study we evaluated the impact of activated fibroblast-derived signals on the resistance and sensitivity to oxaliplatin and cetuximab of colorectal adenocarcinoma cells, mediated by Snail1 and ECM remodeling.

2. Materials and methods

2.1. Cell lines and primary cultures

2.1.1. Patients and colorectal tissue-derived fibroblasts

Human samples were collected after the subjects signed informed consent. Associated clinical information was collected when needed, following the standards of the General Data Protection Regulation in Europe. Samples and clinical data collection processes were approved by the Research Ethical Board at the Ramón y Cajal Hospital (Madrid).

For fibroblast primary culture establishment, the protocol previously published by our group was followed (Herrera et al., 2016). Briefly, fresh CRC tissue samples were cut and seeded in FBS (Fetal Bovine Serum, Corning) with a high concentration of antibiotics (200 U/ml penicillin/200 μg/ml streptomycin, 100 μg/ml gentamicin, 2.5 μg/ml amphotericin b). When the first fibroblasts appear, this medium is replaced by FBM (Fibroblast Basal Medium, Lonza) for cell maintenance. Fibroblasts derived from tumor CRC samples are known as CAFs (Cancer Associated Fibroblasts); and fibroblasts obtained from normal paired tissue will be called NFs (Normal Fibroblasts). Established human primary Cancer Associated Fibroblasts and Normal Fibroblasts from tumor and normal colon and rectal mucosa are short-term cultures of human tumors.

2.1.2. Cell lines

SW480-adherent (SW480-ADH) and DiFi cell lines are derived from human CRC.

SW480-ADH (Pálmer et al., 2001) cell lines were obtained from Alberto Muñoz Laboratory. DiFi and BJhTERT cells were obtained from commercial provider (American Type Culture Collection). Snail1 wt or KO Mouse Embrionic Fibroblasts are derived from a mouse bearing a Snail1-floxed form of this gene and were obtained from Antonio García de Herreros Laboratory (Stanisavljevic et al., 2015). MEFs were used at low passages and without experimentally induced or spontaneous immortalization.

The cell line SW480-ADH was used for oxaliplatin assays and DiFi cells were used for cetuximab experiments, based on their susceptibility to each drug. DiFi cells present EGFR gene amplification without alterations in KRAS, BRAF and PIK3CA, paralleling the molecular features of the CRC patients most likely to respond to cetuximab (Untawale et al., 1993; De Roock et al., 2010) [https://depmap.org/portal/cell_line/ACH-002233?tab=mutation]; showing a high sensibility to anti-EGFR (Lu et al., 2016). Fibroblast cell lines BJ-hTERT (human) and MEF (Mouse Embryonic Fibroblasts), either wild-type or KO for *Snai1*, were used for extracellular matrix generation.

Cell lines were grown in Dulbecco's modified Eagle medium (DMEM, Corning), supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml Normocin and 0.25 μg/ml amphotericin B. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and routinely tested for authentication.

2.2. Extracellular matrices

3D-Matrices derived from fibroblast (either WT MEF, *Snai1* KO MEF or primary fibroblasts from colorectal tissue) were generated as described by our group (Galindo-Pumariño et al., 2019) and adapted (Castelló-Cros and Cukierman, 2009). Briefly, fibroblasts were cultured until confluence was reached. Culture plates were treated with 0.2% gelatin (Sigma) solution for 1 h at 37°C. Then, 1% glutaraldehyde (Sigma) was added and incubated for 30 min at room temperature. Glutaraldehyde was washed in PBS (Phosphate Buffered Saline, Corning) and 1 M ethanolamine (Sigma) was added and incubated similarly. Plates were washed again in PBS, fibroblasts were seeded at 5×10^5 cells per well and incubated in DMEM medium with 10% FBS. To increase matrix deposition, 50 μg/ml ascorbic acid (Sigma) was added to the media and changed every other day until day 8. Resulting 3D matrices

were decellularized and blocked with heat-denatured 2% BSA (Bovine Serum Albumin, Sigma).

2.3. Drug assay

The CRC cell lines SW480-ADH and DiFi were seeded onto fibroblast-generated matrices at 3×10^5 cells per well and incubated in 10% FBS DMEM at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, 1% FBS DMEM was added and cells were incubated in starving conditions for another 24 h. Then, drug was added to culture and cells were incubated for 48 h. Medium was then removed and cells were cultured 1 h at 37°C with a molecular probe (CellTracker™ Green CMFDA, Invitrogen) to incorporate fluorescence labeling, following manufacturer's instructions. Finally, cell fluorescence was measured (Ex. 492 nm/Em. 517 nm) in Varioskan™ LUX multimode microplate reader (Thermo Scientific) to test survival. Drug concentration used in SW480-ADH experiments was 5 μM oxaliplatin and 0.5 μg/ml cetuximab, which was added to DiFi cultures, based on IC_{50} screening. Measures were standardized using the fluorescence signal of untreated cells.

2.4. PCR array analysis

PCR Array of genes involved in drug resistance and cancer (PAHS-004Z, Qiagen) was performed in order to study deregulated genes in tumor cell lines seeded either on *Snai1* WT- or KO MEF-derived matrices.

SW480-ADH and DiFi cells were recovered from matrices after 1 h incubation with dispase (1 U) at 37°C. Cells were collected by centrifugation at 1000 rpm for 5 min. RNA was extracted from cell pellet by RNeasy mini kit (Qiagen), performing on-column digestion following manufacturer's instructions. cDNA was obtained by reverse transcription using RT2 First Strand kit (Qiagen) according to manufacturer's instructions. Real-time PCRs were performed in a Light Cycler 480 (Roche) using RT2 SYBR Green PCR Mastermix (Qiagen), following manufacturer's instructions. PCR mix was added to PCR array plates together with the corresponding sample, so that each cDNA sample comprises a full plate. PCR protocol consisted of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C performing fluorescence data collection. Qiagen online tool "Gene Globe RT2 Profiler PCR Data Analysis" (https://geneglobe.qiagen.com) was used to analyze PCR array results. All samples passed software quality control tests regarding reverse transcription efficiency, genomic DNA contamination and array reproducibility. To standardize uploaded data, "automatic selection from full panel" was selected. This software automatically selects a set of genes as reference genes that differ as little as possible from the ones that are tested in the array. Gene Fold Regulation cut-off was set for selection of downregulated genes (Fold Regulation <0) or upregulated ones (Fold Regulation >2).

2.5. Gene set enrichment analysis and gene ontology

Deregulated genes were studied for gene set enrichment analysis (GSEA) to determine the main pathways or biological processes that may be altered. Gene Ontology was studied with the GSEA gene set investigation tool (http://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp), and comprised the categories Biological Process, Cellular Component and Molecular Function. FDR q-value <0.01 was considered statistically significant.

2.6. SNAIL expression in CAFs and NFs

RNA was extracted from primary CAFs and NFs using the TRIsure™-based protocol. RNA was reverse transcribed to cDNA by the cDNA Reverse Transcription kit (Qiagen). Real-time PCR tested *SNAI1* expression levels standardized by human *SDHA* expression as housekeeping gene. PCR reactions were performed in a LightCycler 480 (Roche) using SYBR Select Master Mix (Applied Biosystems) and primers for the human genes *SNAI1* (forward: 5' CACTATGCCGCGCTCTTTC-3'; reverse: 5' GGTCGTAGGGCTGCTGGAA-3') and *SDHA* (forward: 5' TGGGAACAAGAGGGCATCTG-3'; reverse: 5' CCACCACTGCAT-CAAATTCATG-3'). PCR protocol consisted in 45 cycles of 15 s at 95°C followed by 1 min at 60°C, acquiring fluorescence at the end of each cycle. Finally, melting curve was obtained for assesing single product obtention on each PCR reaction.

2.7. TCGA colorectal cancer cohort

SNAIL gene expression information (mRNA expression z-scores relative to diploid samples (RNA Seq V2 RSEM) was obtained from The Cancer Genome Atlas (TCGA) in a colorectal cancer cohort ($N = 531$). Additional clinical information of CRC patients was also collected: stage (I–IV), age, sex of the patients, tumor location and 60-month survival. Each sample was assigned to subgroups depending on *SNAI1* expression z-score: low, medium or high. The *SNAI1* expression cut-off was set at low expression (minimum value of expression to 33%), medium (from 33 to 66% of expression value) and high (from 66% to maximum). Survival data were analyzed with the Kaplan-Meier method for *SNAI1* expression; and the log-rank test studied significance between the groups. Finally, the Cox analysis identified independent prognostic variables.

2.8. Statistical analysis

In-vitro experimental data were contrasted using the Student's *t*-test. All *t*-tests were performed after evaluation of equality of variance with Levene's test. Two-tailed *p*-values ≤ 0.05 were taken as giving statistical significance. All statistical results are derived from 2 to 4 independent experiments, each performed in duplicate.

Association between survival conferred on tumor cells by NF- and CAF-derived matrices and SNAI1 expression was studied by Pearson correlation test. The predictor value of SNAI1 expression in colorectal cancer patients' was analyzed by Kaplan-Meier and Cox tests.

All statistical analysis used the SPSS statistical package, version 14.0.

3. Results

3.1. Extracellular matrices derived from immortalized fibroblasts protect tumor cells treated with oxaliplatin and cetuximab

SW480-ADH cells are known to be sensitive to oxaliplatin, while DiFi cells are sensitive to cetuximab (Myers et al., 2012; Slater et al., 2018). Thus, these cell lines were treated with different concentrations of the corresponding drugs to determine their sensitivity to these drugs by calculating the value of the concentration at which 50% cell death (IC50) occurred (Supplementary Fig. 1).

To study the possible effect of ECM on tumor cell survival, CRC cells were seeded either on fibroblast-derived matrices or non-treated culture plates. SW480-ADH and DiFi cell survival was measured after 48 h 5 μM oxaliplatin and 0.5 μg/ml cetuximab treatment, respectively. Cells were seeded onto BJhTERT-derived matrices and culture plates to observe the effect of the ECM. Although there were no statistical differences, SW480-ADH and DiFi cells showed a trend to increased survival when seeded in BJhTERT-derived matrices rather than cell plates alone (Supplementary Fig. 2A). No difference were observed in SW480-ADH cells survival seeded on *Snai1* WT MEF-derived matrices or paltes alone. However, an statistical difference is observed in the increase of DiFi survival seeded on *Snai1* WT MEF-derived matrices regarding cells seeded directly in the plates. Moreover, the ECM effect on tumor cell proliferation was also studies, but no clear effects were observed (Supplementary Fig. 3).

These data corroborate the ECM's protective action against the cancer therapies oxaliplatin and cetuximab, as expected according to the published literature (Holle et al., 2016; Eke et al., 2013; Keeratichamroen et al., 2018; Dominijanni et al., 2020; Hoshiba and Tanaka,

1863).

3.2. Extracellular matrices derived from Snai1 KO MEF reduce survival in colorectal cancer cells than matrices derived from WT MEF

Due to the involvement of SNAIL in CAF activation as well as in the ECM remodelation previously demostrated by our group (Herrera et al., 2014; Herrera et al., 2018), *Snai1* WT and KO MEF cells were used to study the role of *Snai1* gene expression in survival conferred by fibroblast-derived matrices. Matrices were generated by mouse fibroblasts (either wild-type or knock-out) for *Snai1* gene; and SW480-ADH and DiFi cells were seeded. Tumor cells were treated with oxaliplatin or cetuximab for 48 h and survival was measured by fluorescence. Fig. 1A shows that SW480-ADH cells had similar survival levels when seeded in WT MEF and in *Snai1* KO-derived matrices without drug treatment. However, when 5 μM oxaliplatin was added to the cell culture, resistance to drug was lower in cells seeded onto *Snai1* KO-derived matrices. This indicates that fibroblasts' *Snai1* expression has a protective role against oxaliplatin treatment. On the other hand, DiFi proliferation showed differences between *Snai1* WT- and KO-derived matrix cultures when the drug was not added. As with oxaliplatin administration, DiFi cells' survival was lower on seeding in *Snai1* KO-derived matrices when 0.5 μg/ml cetuximab was added (Fig. 1B).

3.3. CAF-derived matrices induce higher survival in colorectal cancer cells than NF-derived matrices do

To verify the role of CAF-derived ECM in drug resistance NF and CAF human primary cells were established from tissue explant of surgical CRC patients. Fresh CRC tissue samples were obtained, both from tumor and normal CRC tissue. After primary fibroblasts were obtained, matrices were then generated and SW480-ADH or DiFi tumor cells were seeded. These tumor cells were treated with oxaliplatin or cetuximab, respectively, and survival was measured as described above. In this study, fibroblasts were obtained from paired tissue samples of 16 patients. As shown in Fig. 2A, with oxaliplatin treatment more SW480-ADH tumor cells survived when seeded on CAF-derived matrices than on NF-derived matrices ($p = 6.63E-03$). A similar trend was observed in cetuximab DiFi-treated cells (Fig. 2B).

After studying drug resistance conferred by CAF- and NF-derived matrices, available paired CAF/NF samples from the same patients were analyzed. Thus, we obtained 4 paired cases in SW480-ADH studies and 3 paired cases for DiFi tests. As shown in Fig. 2C, tumor cells had higher survival levels when seeded in CAF-derived matrices than in NF ones. However, there were notable differences between paired cases, as we can see in SW480-ADH in CAF-NF "pair 61" (4% survival increase), "pair 52" (25% survival increase); and in DiFi experiments in "pair 45" (4.5% survival increase) and "pair 61" (42% survival increase) (Fig. 2D). Differences in survival suggest that fibroblast-derived matrices differ in composition or distribution of components.

3.4. Snail1 expression in fibroblasts modulates ECM-effects on drug resistance

To investigate whether primary fibroblast-derived matrix-conferred survival is associated with fibroblast *SNAI1* expression, RNA was extracted from CAF and NF cells and *SNAI1* gene expression was measured by real-time PCR. *SNAI1* gene expression was then compared to previously studied tumor cell survival seeded in CAF-/NF-derived matrices and treated with oxaliplatin or cetuximab. Pearson's test analysis showed positive correlation between fibroblasts' *SNAI1* expression levels and tumor cell survival. Thus, SW480-ADH cells seeded on CAF/NF matrices and treated with oxaliplatin showed a trend to positive correlation with CAF/NF *SNAI1* expression (Fig. 3A). DiFi cells studied under the same conditions and treated with cetuximab showed positive and statistically significant correlation with CAF/NF *SNAI1* expression ($p = 0.007$) (Fig. 3B). These results suggest that fibroblasts' *SNAI1* expression is related to matrix-conferred survival.

3.5. SNAI1 expression in fibroblasts regulates drug resistance and metabolism gene expression in tumor cells mediated by the ECM

To analyze genes or pathways that are involved in chemoresistance to tumor cell lines SW480-ADH and DiFi provided by fibroblast-derived matrices, PCR array studies were performed. Expression of genes relating to cancer and drug resistance was studied with pre-designed PCR array plates (PAHS-004Z, Qiagen), including DNA repair genes, cell cycle regulators and growth factor receptor genes, among others (see full 84-gene list in Supplementary Table 1). In this way, SW480-ADH and DiFi cells were seeded on WT or *Snai1* KO MEF-derived matrices and treated with oxaliplatin and cetuximab respectively. Then, tumor cells SW480-ADH and DiFi were recovered from MEF-derived matrices and total RNA was extracted and retrotranscribed for PCR array assays. Enrichment in gene ontology categories was then performed.

PCR array gene expression showed that 40 genes were upregulated and 12 genes were downregulated in oxaliplatin treated SW480-ADH cells when cells were seeded on *Snai1* KO MEF-derived matrices regarding cells seeded on WT MEF-derived matrices(Fig. 4A). Functional enrichment analysis was conducted for downregulated genes (Fold Regulation <0) in SW480-ADH cells seeded onto *Snai1* KO MEF. Thus, genes with direct association with fibroblast *Snai1* expression are

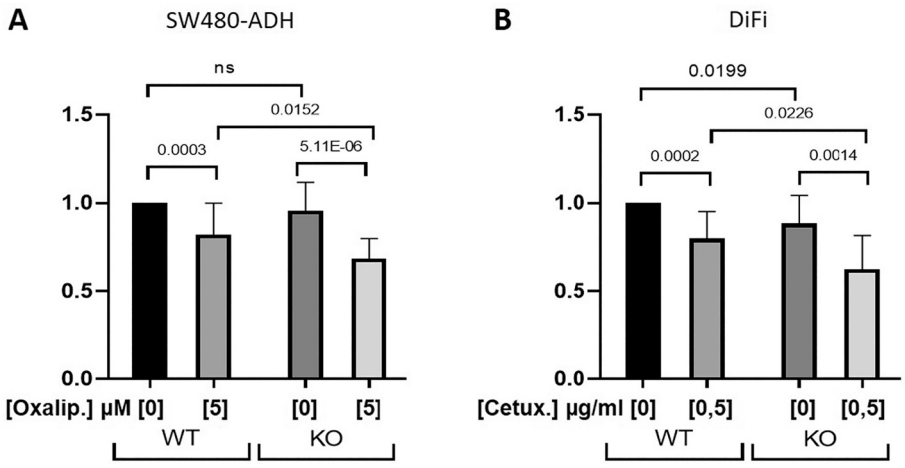

Fig. 1. Extracellular matrices derived from Snai1 KO MEF reduce survival in colorectal cancer cells when treated with oxaliplatin and cetuximab more than matrices derived from WT MEF do. A) SW480-ADH cells' survival seeded on ECM, derived from WT MEFs or Snai1 KO MEFs and treated with oxaliplatin for 48 h (concentration is shown in brackets). B) DiFi cells' survival seeded on ECM, derived from WT MEFs or Snai1 KO MEFs and treated with cetuximab for 48 h (concentration is shown in brackets). Survival was normalized using cells seeded onto WT MEF's derived matrix without drug treatment as control.

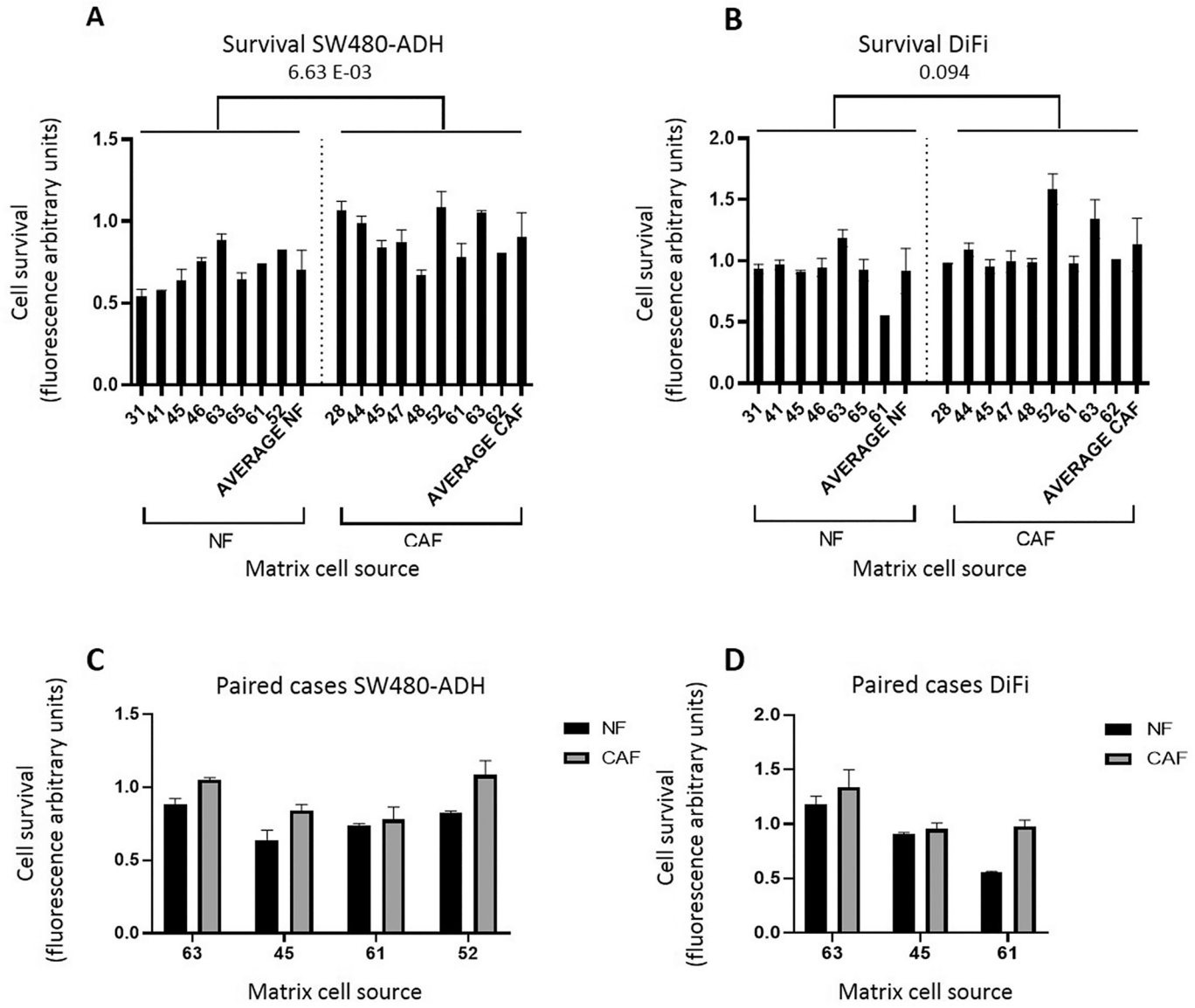

Fig. 2. CAF-derived matrices confer more resistance on tumor cells than NF-derived matrices do, under oxaliplatin and cetuximab treatment. A) SW480-ADH cells' survival seeded on ECM, derived from CAFs or NFs and treated with oxaliplatin. B) DiFi cells' survival seeded on ECM, derived from CAFs or NFs and treated with cetuximab. Average of survival of cells seeded onto NF and CAF-derived matrices measured by fluorescence is shown in A and B. C) SW480-ADH cells' survival seeded on the ECM, derived from CAFs and NFs paired and treated with oxaliplatin. D) DiFi cells' survival seeded on the ECM, derived from CAFs and NFs paired and treated with cetuximab. Cells were treated either with oxaliplatin or cetuximab for 48 h, then survival was measured by fluorescence using cells seeded on normal plates and treated similarly as control. Numbers in X-axes indicate primary fibroblast case, isolated form patient's tissue and codified for confidentiality.

involved in drug and hormone metabolism. Upregulated genes (Fold Regulation >2), inversely associated with fibroblasts' *Snai1* expression, are associated with, response to xenobiotic stimulus, cell death regulation or apoptosis (Supplementary Table 2A). Remarkably, apoptosis signaling is clearly over-regulated in tumor cells seeded onto *Snai1* KO-derived matrices, as expected, after oxaliplatin exposition since several apoptosis-related genes are overexpressed as BCL2L1, BCL2, BAX and RB1. These data indicate a protective role of ECM derived from *Snai1* expressing fibroblasts regarding those derived from *Snai1* KO fibroblast.

PCR array gene expression showed that 22 genes were upregulated and 21 genes were downregulated in cetuximab treated DiFi cells when cells were seeded on *Snai1* KO MEF-derived matrices regarding cells seeded on WT MEF-derived matrices (Fig. 4B). Functional enrichment analysis was conducted for downregulated genes (Fold Regulation <0) in DiFi cells seeded onto *Snai1* KO MEF cell cycle checkpoints and cell death regulation. Upregulated genes (Fold Regulation >2) were associated with changes in response to xenobiotic stimulus, programing and regulation of cell death or nuclear receptor activity (Supplementary Table 2B). Interestingly, EGFR downstream efectors as ELK1 or MET were downregulated in tumor cells seed onto *Snai1* KO-derived matrices after cetuximab exposition pathway. These data indicate a bigger cetuximab toxicity in tumor cells seeded onto *Snai1* KO-derived matrices.

All these results support the role of fibroblasts' *Snai1* expression in tumor cell drug resistance mediated by extracellular matrix.

3.6. Association between tumor SNAI1 expression and survival in a CRC patient cohort

Once the important role of fibroblasts' *SNAI1* expression in ECM-mediated chemoresistance had been established, we studied the potential effect of SNAIL on colorectal cancer patients' survival. In this study, *SNAI1* expression data and relevant clinical information were collected from a colorectal cancer cohort of 531 patients from the TCGA database

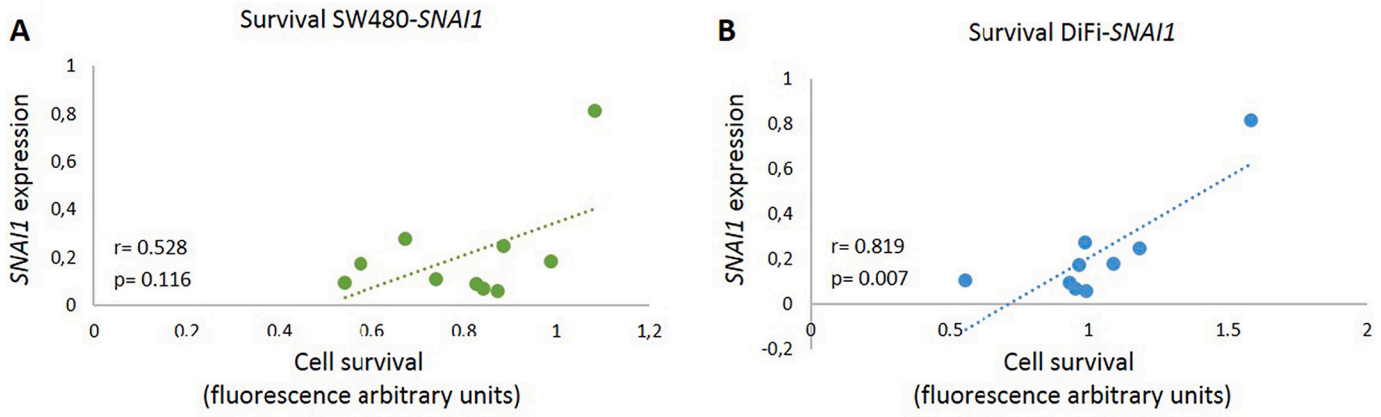

Fig. 3. Survival conferred on tumor cells by NF- and CAF-derived matrices is associated with fibroblast SNAI1 expression. A) Correlation between SW480-ADH cells' survival seeded on the ECM, derived from CAFs or NFs and treated with oxaliplatin, and SNAI1 expression levels in NF and CAF cells. B) Correlation between DiFi cells' survival seeded on the ECM, derived from CAFs or NFs and treated with cetuximab, and SNAI1 expression levels in NF and CAF cells. SNAI1 relative expression was measured by Real-Time PCR using SDHA as housekeeping gene.

(TCGA Research Network; https://www.cancer.gov/). The *SNAI1* expression data collected are summarized in Supplementary Table 3. Patients were divided according to *SNAI1* expression into three groups (low-, medium- and high-expression groups) with a similar number of patients. Kaplan-Meier survival analysis showed that the high- and medium-SNAIL expression groups had lower OS, while the low-expression group showed higher survival (p = 3.02E-04) (Fig. 5A). Since colon and rectal tumors are considered biologically and clinically quite different (Tamas et al., 2015), 60-month survival analysis was then performed separately for rectal and colon location of the tumors, taking into account the low-medium-high *SNAI1* expression groups. Kaplan-Meier survival curves showed stronger prognosis value in colon cancer patients (p = 1.65E-03), while separation between curves was not so different in rectal cancer patients' (p = 0.082) (Fig. 5B).

The Cox analysis also revealed the independent prognostic value of SNAI1 expression, independently of other important clinical parameters such as stage, age, sex or tumor location (rectal/colon) (Table 1). All these data prove the clinical relevance of tumors' SNAI1 expression when calculating survival of CRC patients.

4. Discussion

Currently, clinical decisions are made on the basis of the usual histologic and clinical parameters that do not accurately predict the biologic behavior of histologically identical tumors. Moreover, antitumor therapy resistance is one of the most important problems that clinicians face in patient management. In order to improve patients' response, a great effort must be made to identify predictive biomarkers to implement decission making regarding the therapeutic guidelines to be followed in CRC patients. Thus, one of the biggest challenges is to understand chemoresistance mechanisms in order to apply better treatment lines. Efforts are still being made to study tumor cell properties, even though tumor microenvironment had been identified as mediator in different resistance events (Khalaf et al., 2021). For instance, subclasses of tumor immune microenvironment influence are associated with those tumors more prone to immune-checkpoint blockade responsiveness (Binnewies et al., 2018). ECM proteins that mediate fibrosis are also involved in drug delivery and thus promote resistance against cytotoxic therapies (Gamradt et al., 2021). CAFs, the principal cell component in tumor stroma, release soluble factors that are associated with induced chemoresistance in colon cancer cells (Gonçalves-Ribeiro et al., 2016). Moreover, CAFs are responsible for ECM production and remodeling (Pietras and Östman, 2010; Kalluri, 2016). At the same time, the ECM conditions tumor growth, favoring angiogenesis and cell migration (Herrera et al., 2018; Holle et al., 2016). Currently, increasing evidence points to the ECM's role in mediating resistance to existing treatments, including cytotoxic and targeted therapy drugs (Eke and Cordes, 2015a; Kesh et al., 2020; Leask, 2020). Many studies have shown the involvement of ECM-stiffness in cancer drug resistance (Khalaf et al., 2021; Liu et al., 2020). For instance, ECM-stiffness is positively associated with hepatocarcinoma resistance to paclitaxel, cisplatin, 5-FU and sorafenib (Nguyen et al., 2014; Liu et al., 2015). In addition, the ECM induces chemoresistance by acting as a barrier for drugs and activating tumor cell growth mediated by ECM-tumor cell adhesion. Integrins and FAK complex are involved in cell adhesion mechanisms, revealing a role in drug resistance events (Kalluri, 2016; Eke and Cordes, 2015b). Thus, depending on integrin expression in response to ECM-stiffness, hepatocarcinoma cells upregulate phosphorylation of the Akt/mTOR pathway mediating resistance to oxaliplatin (You et al., 2016). In a similar way, ECM characteristics also mediated 5-fluorouracil resistance in colorectal cancer cells (Hoshiba and Tanaka, 2016). With regard to resistance to targeted therapy drugs, a decrease in cetuximab response induced by tumor cell adhesion to fibronectin has been reported (Eke et al., 2013). Moreover, Rahbari et al. discussed the connection between the increase of ECM in metastatic CRC patients under antiangiogenic therapy and acquired drug resistances (Rahbari et al., 2016). In line with these data, our results show how the fibroblast-derived extracellular matrix alters tumor cell response to both oxaliplatin and cetuximab, cytotoxic and targeted therapy drugs, depending on SNAI1 expression highlighting the biological relevance of our data.

SNAI1 is a zinc finger transcription factor, encoded by the *SNAI1* gene that has a major role in the Epithelial-Mesenchymal Transition. EMT is a cell mechanism characterized by promotion of mesenchymal properties instead of epithelial characteristics favoring cell migration, apoptosis evasion or drug resistance in cancer (Baulida et al., 2019; Assani and Zhou, 2019). Adult fibroblasts express *SNAI1* under pathological situations, such as wound healing or cancer (Stanisavljevic et al., 2015). Previously, our group proposed *SNAI1* expression as a fibroblast activation marker (Herrera et al., 2014), acting in ECM remodeling (Stanisavljevic et al., 2015). Thus, *SNAI1*-expressing fibroblasts induce fiber deposition and degradation in ECM, conditioning matrix remodeling and fiber orientation, including collagen and fibronectin protein filaments (Stanisavljevic et al., 2015; Rowe et al., 2009; Stanisavljevic et al., 2011; Lu et al., 2013). We have also reported that fiber alignment associated with *SNAI1* induces activation of endothelial cells and, thus, tumor neovascularization (Herrera et al., 2018). In the present study, we observed that tumor cells show lower survival levels when seeded onto

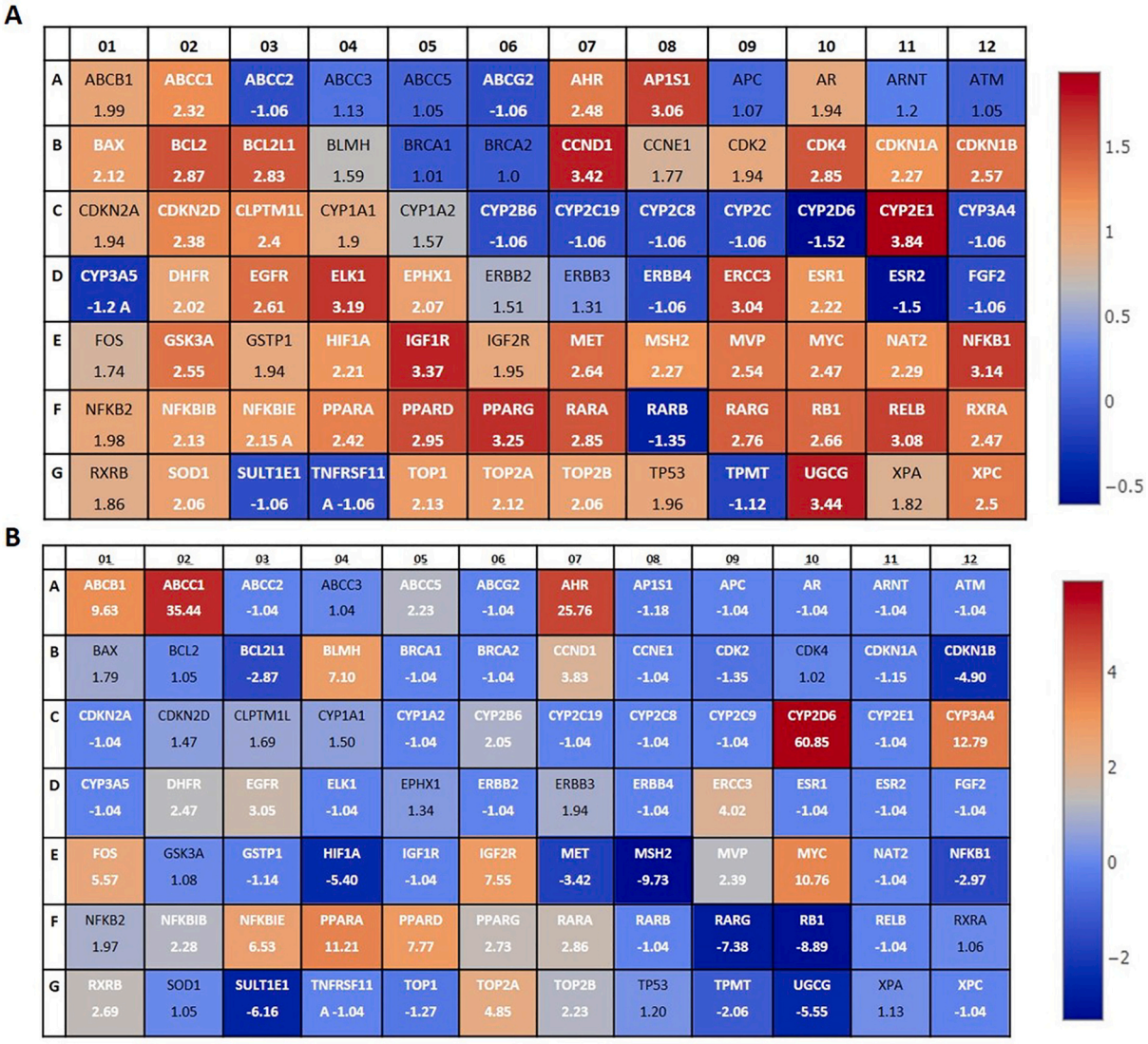

Fig. 4. Snai1 expression in fibroblasts regulates drug resistance and metabolism gene expression in tumor cells mediated by the ECM. A) Deregulated genes obtained by PCR array in SW480-ADH cells treated with oxaliplatin and seeded onto KO MEF Snai1-derived matrices. B) Deregulated genes obtained by PCR array in DiFi cells treated with cetuximab and seeded onto KO MEF Snai1-derived matrices. PCR array gene expression of cells seeded onto MEF WT-derived matrices was used as control. Qiagen tool "Gene Globe RT2 Profiler PCR Data Analysis" was used for PCR array analysis. Genes marked in white (Fold Regulation>2 or < 0) were selected for Gene Ontology analysis.

matrices derived from *SNAI1*-depleted fibroblasts. These data indicate the role of SNAI1-expressing CAFs in drug resistance in colorectal cancer patients mediated by ECM modification.

Due to differences in drug sensitivity in our study, we investigated the pathways that were altered in tumor cells treated with either oxaliplatin or cetuximab and seeded onto *Snai1* WT and KO MEF. We observed different results for the two cell lines studied, which was probably due to the different mechanisms of action of each drug. While oxaliplatin is a cytotoxic drug that forms platinum-DNA adducts inducing cell death, cetuximab is an anti-EGFR targeted agent that interrupts cell proliferation. Thus, cells treated with oxaliplatin revealed alterations in drug response pathways. Additionally, apoptosis and apoptosis regulation pathways are also altered, which confirms our survival studies, in which tumor cells showed lower drug tolerance when they were seeded onto matrices derived from *Snai1*-depleted fibroblasts. Finally, Gene Ontology study also found altered transcription initiation, which can be associated with oxaliplatin-induced DNA adducts. On the other hand, cetuximab treatment affected pathways relating to response to drugs and xenobiotic and organic cyclic compounds. Likewise, transcription activation routes are altered as well as pathways related to cell cycle control and proliferation, which is expected due to the cetuximab mechanism of action, which directly acts on cell proliferation.

CAFs' heterogeneity determines ECM remodeling, MMP secretion and fiber deposition, along with secretion of pro-angiogenic and growth factors (Junttila and De Sauvage, 2013). These differences between fibroblast populations, which are also detected in normal fibroblasts, could modulate drug resistance response in tumors (Kobayashi et al., 2019). In the present study, matrices were generated from CAFs and normal fibroblasts (NFs); and CRC cell lines were seeded and treated with oxaliplatin and cetuximab. Results revealed that tumor cells seeded on CAF-derived matrices were more resistant to oxaliplatin and cetuximab treatments. Paired-cases study indicated that CAF-derived matrices induced higher protection from drugs in all cases, although resistance induced by CAF-derived matrices varied greatly among CAF/NF pairs. It is of interest that the highest values of matrix-conferred resistance occur in fibroblasts from individuals with stage II–III disease, while these values are lower in the matched-case individual with

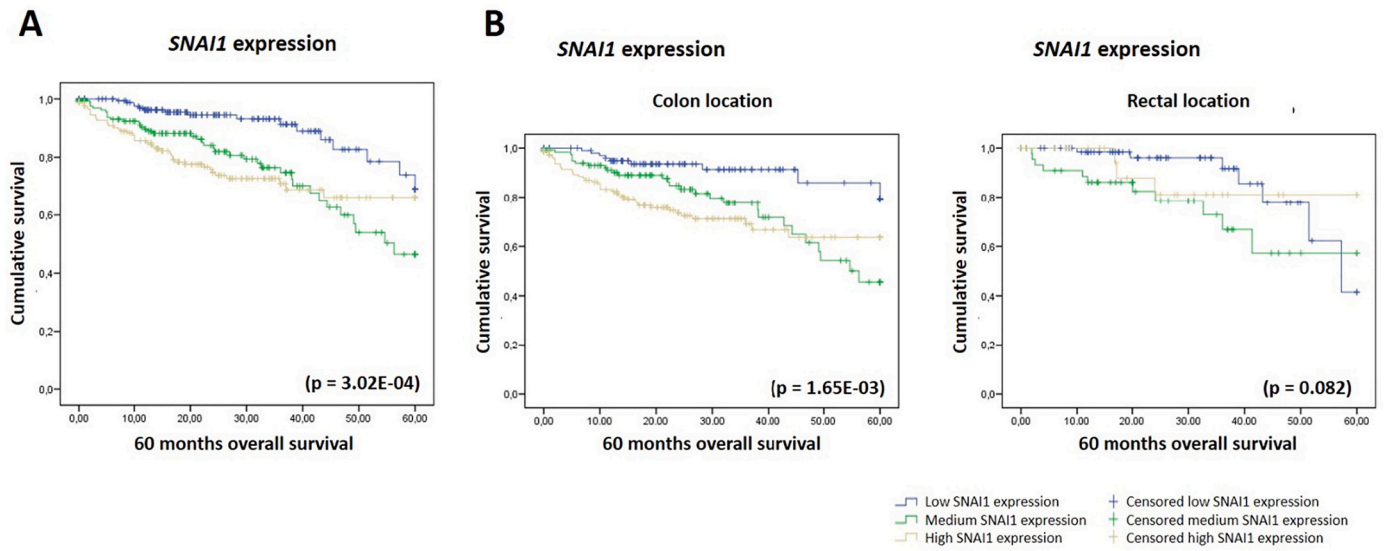

Fig. 5. SNAI1 as a predictor of colorectal cancer patients' prognosis. (A) Kaplan-Meier curves showing the association between variable SNAI1 expression and colorectal cancer patients' 60-month survival. (B) Kaplan–Meier curves showing the association between the expression levels of CAF SNAI1 markers and survival for anatomical location of tumor in colorectal cancer patients.

Table 1
Univariate and multivariate cox analysis of the association between SNAI1 expression and 60 month overall survival on colorectal cancer patients.

Variable	Category	Unadjusted analysis		Adjusted analysis	
		HR (95% CI)	*p*	HR (95% CI)	*p*
Age at diagnosis	<68 vs ≥ 68	2.85 (1.83–4.45)	3741E-06	2.84 (1.83–4.42)	3.50E+08
Stage	I + II vs III + IV	3.53 (2.27–5.51)	24297E-08	3.47 (2.23–5.38)	28831E-08
Sex of patients	Male vs female	0.91 (0.60–1.38)	0.672		
Tumor location	Rectal vs colon	0.85 (0.51–1.42)	0.53998505		
SNAI1 expression	Medium vs low	2.042 (1.13–3.71)	0.01876025	2.08 (1.15–3.77)	0.01504027
	High vs low	2.18 (1.20–3.96)	0.01055563	2.27 (1.27–4.06)	0.00594979

CI, confidence interval; HR, hazard ratio. Blank cells correspond to variables that showed no independent relations to OS in the multivariate analysis.

stage I disease (data not shown). This is consistent with the idea that more advanced and aggressive disease corresponds with a more active microenvironment, and thus the role of the microenvironment in the response to treatment may be more relevant. Moreover, the association observed between SNAI1 expression levels in NFs/CAFs and tumor cell-conferred survival under drug treatment points to SNAI1 expression in fibroblasts as a possible biomarker with predictive value of CRC patients' response to oxaliplatin and cetuximab treatments.

SNAIL has been associated with an increased level of MMP, invasiveness, angiogenesis and metastasis (Stanisavljevic et al., 2015; Peinado et al., 2007). To study SNAIL effects in CRC patients, *SNAI1* expression and 60-month survival data were analyzed in a cohort of 531 patients. It was observed that the high-*SNAI1* expression group resulted in worse survival. Other groups have reported that *SNAI1* levels in stroma, instead of tumor, were associated with survival in early stages (Stanisavljevic et al., 2015; Francí et al., 2006). Our data showed statistical differences in both early and advanced stages. These differences are probably because SNAI1 expression levels come from analysis of the whole tumor and not from the stromal compartment, as was the case in these previous studies. In support of our data, SNAIL expression has also been associated with worse survival in breast, brain and uterus tumors (Chang et al., 2018; Myung et al., 2010; Tian et al., 2020). Interestingly, our data showed no significant association between *SNAI1* expression in primary tumor and rectal location. Actually, rectal and colon carcinomas can be seen as two different types of cancer. Moreover, the standard treatment schedules are quite different for both pathologies. This is seen particularly in the radiotherapy treatment prior to surgery in rectal tumors, which is not usually done in colon tumors (Tamas et al., 2015). Probably, these treatment differences directly affect the role of SNAIL in tumor progression. Thus, based on all the data given, we can deduce that SNAIL could be a predictive biomarker of oxaliplatin and cetuximab therapy response in colon cancer indicating the clinical relevance of our study.

Although there is a lot of information on the importance of the microenvironment in the development of resistance to antitumor treatments, no stromal biomarkers are used in clinical practice to indicate the treatment guidelines to be followed in the management of oncological patients. The data given in this study show that the expression of SNAI1 in fibroblasts modulates the resistance of CRC cells to oxaliplatin and cetuximab, mediated by communication with the ECM. In addition, it is important to point out the prognostic value of SNAI1 expression in patients with colon cancer, which, together with in vitro experimentation, suggests that SNAI1 expression in tumor-associated fibroblasts could be used as a predictive biomarker of response to oxaliplatin and cetuximab treatments in patients with colon cancer (Fig. 6). Assistant treatment decisions are made based on the usual histological and clinical parameters that do not accurately predict the patients' response and evolution of the disease. Adjuvant chemotherapy in stage III patients has been clearly demonstrated to benefit patients with stage III disease, increasing the 5-year disease free survival from 49.0% to 63.6% (Gonçalves-Ribeiro et al., 2016). Oxaliplatin plus a fluoropyrimidine treatment during 6 months is the standard adjuvant therapy for stage III CC patients. Otherwise, cetuximab is indicated for the treatment of K-Ras wild-type, epidermal growth factor receptor (EGFR)-expressing, metastatic colorectal cancer as determined by an FDA-approved test (Fda, 2022). Although both shown benefit in patients survival, it is important to note

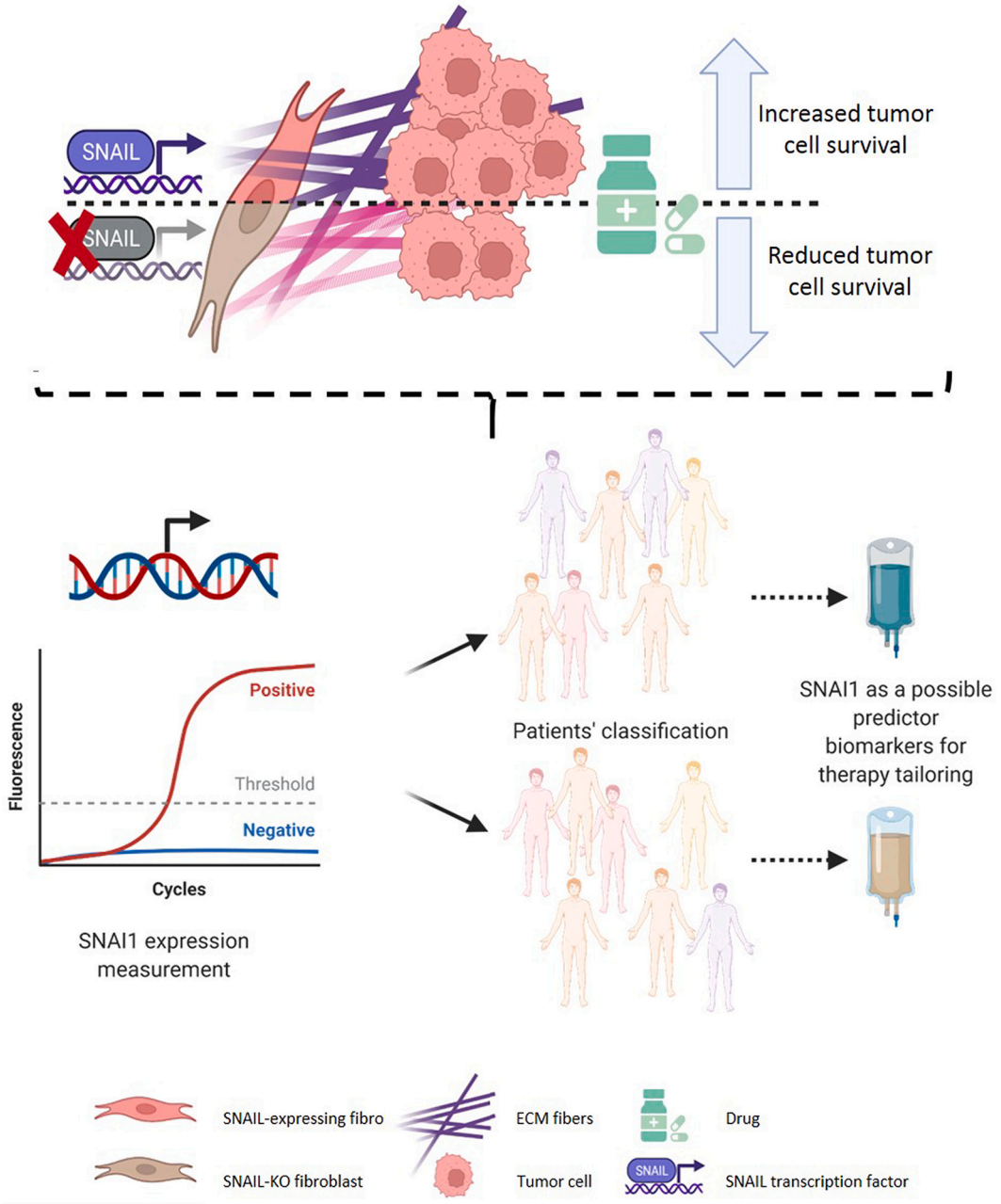

Fig. 6. Fibroblast-derived matrix affects tumor cell response to chemotherapy. Fibroblasts' SNAI1-expression conditions the response of tumor cells to oxaliplatin and cetuximab drug, mediated by the ECM. Presumably, SNAI1 expression in tumor-associated fibroblasts could be used as a new clinical tool to predict the response to oxaliplatin and cetuximab treatments in patients with colon cancer. Created with BioRender.com

the important side effects of these treatments. These effects may be avoided whether the patients do not present any response to the treatment. Thus, oxaliplatin side effects include sensory neurotoxicity (depending on accumulated doses) which can be severe and affects patients' activities of daily living for the rest of their lives (Grothey, 2003). Cetuximab side effects include, infusion reactions, cardiopulmonary arrest, pulmonary toxicity, dermatologic toxicity and hypomagnesemia (Fda, 2022). Moreover, its use is not indicated in combination with radiation and cisplatin or in patients with Ras mutations since the treatment resulted in no clinical benefit with related toxicity. In a similar way, the data from our study, including in-vitro experimentation and clinical data, show the expression of SNAI1 as an indicator of oxaliplatin and cetuximab failure treatment in CRC patients. Due to the side effects of these two treatments, it is clear the need to search for new biomarkers that indicate whether patients will respond or not to the treatment. Thus, SNAI1 expression could be an indicator of treatment failure and thus, although more studies might confirm this observation, it could be presented as a new tool for treatment making decisions regarding oxaliplatin and cetuximab in CRC patients. An obvious clinical implication of these findings is that those patients with SNAI1 expression could be beneficiaries of salvage therapies most adequate to their molecular cancer progress with presumably better response and improvement in their survival and quality of life. In addition, our data lay the foundation to continue searching for new therapies to target microenvironment tumor cells and emphasizes the ongoing efforts to identify treatment response biomarkers in CRC patients in an attempt to implement personalized medicine to treat the individualized patients and not the disease in an standard way.

Author contributions

All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Research Ethics Committee of "Hospital Universitario Ramón y Cajal" (protocol code CEIC_008-18 and 1/29/2018).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

C. Galindo-Pumariño: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **M. Collado:** Methodology, Investigation. **M.E. Castillo:** Methodology, Investigation, Resources. **J. Barquín:** Resources. **E. Romio:** Resources. **M.J. Larriba:** Methodology, Investigation. **G.J. Muñoz de Mier:** Investigation. **A. Carrato:** Writing – review & editing, Funding acquisition. **C. de la Pinta:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **C. Pena:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2022.116171.

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