FUNCTIONAL HETEROGENEITY OF CANCER-ASSOCIATED FIBROBLASTS
FROM HUMAN COLON TUMORS SHOWS
SPECIFIC PROGNOSTIC GENE EXPRESSION SIGNATURE

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Running title: Prognostic CAF signature in colon cancer

Keywords: Cancer-Associated Fibroblasts; Colon Cancer; Gene expression profile; Patient survival.
Financial support: This study was supported by the Fundación Científica AECC, PI12/02037, SAF2010-20750, CM-S2010/BMD-2344, RD12/0036/0041, and the Fundación Banco Santander. Antonio García de Herreros’ laboratory was supported by RD12/0036/0005. Cristina Peña is a recipient of a Miguel Servet Contract from the Instituto de Salud Carlos III (CP09/00294); and Vanesa García, of a contract from AECC.

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All authors state no conflicts of interest.
STATEMENT OF TRANSLATIONAL RELEVANCE

Findings reveal, for the first time, the heterogeneity, among different patients, of activated cancer-associated fibroblasts effect on cancer cells. This fibroblast heterogeneity defines a gene expression signature able to classify colon cancer patients into high- and low-risk groups, with important implications: firstly, in the ongoing efforts to identify colon cancer cell prognostic markers in colon cancer progression. And secondly, emphasizes the search of new treatments to arrest fibroblasts-epithelial interactions to interfere colon cancer progression.
ABSTRACT

Purpose: Cancer-associated fibroblasts (CAFs) actively participate in reciprocal communication with tumor cells and with other cell types in the microenvironment, contributing to a tumor-permissive neighborhood and promoting tumor progression. The aim of this study is the characterization of how CAFs from primary human colon tumors promote migration of colon cancer cells. Experimental Design: Primary CAF cultures from 15 primary human colon tumors were established. Their enrichment in CAFs was evaluated by the expression of various epithelial and myofibroblast specific markers. Co-culture assays of primary CAFs with different colon tumor cells were performed to evaluate pro-migratory CAF-derived effects on cancer cells. Gene expression profiles were developed to further investigate CAF characteristics. Results: Co-culture assays showed significant differences in fibroblast-derived paracrine pro-migratory effects on cancer cells. Moreover, association between CAFs’ pro-migratory effects on cancer cells and classical fibroblast activation or stemness markers was observed. CAF gene expression profiles were analyzed by microarray to identify deregulated genes in different pro-migratory CAFs. The gene expression signature, derived from the most pro-tumorogenic CAFs, was identified. Interestingly, this “CAF signature” showed a remarkable prognostic value for the clinical outcome of colon cancer patients. Moreover, this prognostic value was validated in an independent series of 142 colon cancer patients, by RT-qPCR, with a set of four genes included in the “CAF signature”. Conclusions: In summary, these studies demonstrate for the first time the heterogeneity of primary CAFs’ effect on colon cancer cell migration. A CAF gene expression signature able to classify colon cancer patients into high- and low-risk groups was identified.
INTRODUCTION

Tumor epithelial cells within a tumor coexist with a complex microenvironment. In this microenvironment, extracellular matrix, growth factors, cytokines and a variety of non-epithelial cell types, including vascular space-related cells (endothelial cells, pericytes and smooth muscle cells), inflammatory response cells (lymphocytes, macrophages and mast cells) and fibroblasts, come together (1). In this complex scenario, a variety of interactions take place between its various components and determine a series of events, such as tumor cell proliferation, metastatic potential and location of metastases (2, 3).

Fibroblasts are one of the most active cell types of the stroma. They form the basic cell component of the connective tissue that contributes to structural integrity, as well as the maintenance of the extracellular matrix (4). In addition, fibroblasts from normal tissues perform tissue repair functions under certain physiological conditions (5). Fibroblasts of the tumor stroma have received various names: tumor-associated fibroblasts, carcinoma-associated fibroblasts (CAFs) or myofibroblasts (6). They are similar to those found in the wound-healing process, although CAFs are constantly activated (6). In this state of permanent activation, fibroblasts promote tumor growth and tumor progression, favoring a variety of tumor-specific mechanisms (7). Thus, CAFs are involved in various tumor mechanisms, such as extracellular matrix remodeling, release of soluble factors, regulation of tumor cell motility and the specialization of tumor metabolism or tumor cell implantation (8-12).

CAFs’ activated phenotype is characterized by expression of different proteins, such as α-SMA (α-Smooth Muscle Actin), FAP (Fibroblast Activation Protein), FSP1 (Fibroblast-
Specific Protein 1) growth and angiogenic factors. However, CAFs comprise a heterogeneous population of cells and are derived from different sources (13). The CAF heterogeneity observed may reflect the variation of CAF involvement in cancer progression.

In this study we established experiments to test the capability of primary colon CAFs to promote tumorigenesis of colon cancer cells and found that CAFs work in paracrine fashion in this direction to augment cancer cell migration. The differential pro-migratory capacity of CAFs identified a gene expression signature that allows classification of colon cancer patients into high- and low-risk groups.
METHODOLOGY

Establishment of primary colon CAFs and Normal Fibroblasts (NFs)

Fresh tissue from 15 patients operated for colorectal primary tumors at the Puerta de Hierro-Majadahonda University Hospital was used for the propagation of primary CAFs and NFs. Informed written consent was obtained from all participants, as required and approved by the Research Ethics Board of the Puerta de Hierro-Majadahonda University Hospital. For CAF and NF establishment and growth details, see Supplementary Information.

Determination of fibroblast senescence and immunofluorescence

Immunofluorescence analyses followed standard procedures with monoclonal anti-pan cytokeratin (ab6401, Abcam), monoclonal anti-vimentin (ab92547, Abcam) and monoclonal anti-alpha smooth muscle actin (ab7817, Abcam). For details, see Supplementary Information.

To characterize colon fibroblasts, their senescence status was evaluated by the use of the Senescence β-Galactosidase Staining Kit (Abcam), in line with the manufacturer's instructions (details in Supplementary Information).

Collagen gel contraction assay

Collagen gels were prepared with Type I collagen (1.5mg/mL, Rat Tail Tendon Collagen, RTTC) (BD Biosciences), DMEM (supplemented with penicillin and streptomycin, 200 units/mL, and 2 mM L-glutamine), gel contraction buffer (20 mmol/L HEPES, 0.22% NaHCO₃, 0.005N NaOH), 20% FBS and CAF cells. The mixture (500µL) was cast into each well of a 24-well culture plate. The solution was then allowed to polymerize at 37°C for 30
min. After polymerization, the gels were gently released from the plate and transferred into 100-mm tissue culture dishes. After 24h, the surface area of the gels was measured. The values of the fibroblast gels were normalized to that of the control gel without cells.

Co-culture of tumor colon cells and human colon primary CAFs or NFs for migration assays

Colorectal tumor cell lines LIM1215 or SW480-ADH were co-cultured with colon primary CAFs or NFs for migration assays. Cells were co-cultured with physical separation, in a setting in which primary CAFs or NFs were seeded in the lower compartment of a transwell system and colon cells in the upper one (Corning Incorporated, Costar, #3428). After 48 h, epithelial cells that had reached the lower surface of the filter were measured by fluorescence.

Osteoblast Differentiation Assay

CAF were differentiated by adding human Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza, PT-3002) to a confluent culture of fibroblasts for 16 days, following the manufacturer’s protocol, while CAF control cells were maintained with FBM. Osteoblast differentiation is marked by the formation of mineralized nodules composed of inorganic hydroxyapatite and other organic components. Thus, mineralization was evaluated with the Osteolmage Mineralization Assay (Lonza, PA1503) in line with the manufacturer’s instructions, and quantitatively measured by a plate reader (Tecan Infinite 200Pro.).

Affymetrix Gene Chip Hybridization
Total RNA was extracted from CAF samples with TRIzol reagent (TRIzol, Invitrogen-Gibco), in line with the manufacturer’s protocol. In accord with the Affymetrix protocol (Affymetrix expression manual), all samples were processed and 2ug of fragmented and labeled cDNA were hybridized to the Affymetrix GeneChip arrays (Human Gene 1.0 ST). See Supplementary Information for further details.

Microarray data analysis

Microarray data were normalized and differential expression (DE) analyses were conducted, using Bioconductor (14) packages, “affy” (15) (version 1.28.1) and Limma (16), respectively. Raw data and normalized microarray expression data were submitted to GEO database under the accession number GSEXXXX" (accession number will be provided with finally approved manuscript). To determine the DE profiles of CAF, the expression value from two NF sample averages was subtracted from every CAF sample. For “significant” DE genes, we set the p-value cut-off ≤ 0.05 and the Log2 fold change cut-off at 0.5. Gene Ontology (17) (www.geneontology.org) functional enrichment analysis and heat-map viewing of distinctly expressed genes were based on the Gitoools program (18) (www.gitoools.org). Using microarray expression data, we determined with ANOVA statistics the CAF gene signatures that were significantly altered (p value ≤ 0.05) in “gene-normalized” (see Supplementary Information for details) expression data, when comparing higher versus lower migratory groups.

Public microarray data and survival analysis

A cohort of 232 colon cancer patients from the GEO database (GEO accession GSE17538) was used to assess the prognostic value and clinical relevance of “CAF signatures” in colon
cancer. Public microarray data on colon cancer were normalized as described above. We then used Gitools for Sample Level Enrichment Analysis (SLEA) (19) with modular Z-score enrichment statistics (18). Positive and negative z-scores indicated significantly higher or lower expression levels of genes in the module (CAF signature and Golgi genes), respectively. We grouped the colon cancer samples according to their modular expression; and Kaplan-Meier survival curves and the Cox Hazard Ratio (HR) were then calculated with the R statistical program package, “survival” and “survplot”.

**Quantitative Real-Time PCR (RT-PCR)**

RNA extraction, target gene mRNA quantification and synthesis of cDNA were performed as described elsewhere (20).

The sequences for PCR primers are listed in Supplementary Table S1.

At the end of the PCR cycles, melting curve analyses were performed in order to confirm the specificity of the amplification reactions. The PCR products were sequenced in an ABI Prism™ 377 DNA sequencer apparatus (PE Applied Biosystems).

**Statistical analysis**

Comparisons between gene expression levels, collagen contractile abilities in different promigratory CAFs and between CAF and NF migration effects on cancer cells 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.
Patients and analysis of “CAF signature” validation

The study is based on a consecutive series of 142 patients undergoing surgery for colon cancer between January 2002 and December 2006. Informed written consent was obtained from all participants, as approved by the Research Ethics Board of Puerta de Hierro Majadahonda University Hospital. Colon cancer-derived samples were obtained immediately after surgery, snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until processing.

The number of patients in which the detection of the target gene was possible and percentile data of each gene mRNA quantification, is detailed in Supplementary Table S2. mRNA expression data of these genes were divided by quartiles (25\%, 50\% and 75\%) to get four groups of patients and develop the statistical analysis of Kaplan-Meier survival curves and the Cox Hazard Ratio (HR), as previously described. Statistical analysis revealed similar behavior of different gene expression data quartiles of each gene. Therefore, to simplify the analysis, those quartiles were grouped according to mathematical behavior and thus, the variables were dichotomized into “High” and “Low” gene expression levels.
RESULTS

Primary CAFs isolated from colon tumors show activated fibroblast features

Fresh tissue from primary tumors of 15 patients operated on for colorectal cancer in our hospital was used for the propagation of primary CAFs, as described in the Methodology section (Figure 1A). Then, morphology and immunohistochemistry were characterized to assess fibroblast characteristics. Fibroblast cultures maintained the phenotypic properties of activated fibroblast or myofibroblast even in absence of interaction with carcinoma cells. Primary CAFs initially showed bi- and/or multi-polar morphology and then acquired a uniform spindle-shaped morphotype and formed parallel arrays and whorls at confluence. Moreover, expression of α-sma in primary CAFs was also confirmed (Figure 1B). To evaluate the CAF enrichment of the culture, Vimentin and Pan Cytokeratin (PanCK) were analyzed for immunofluorescence (Figure 1C). CAFs were studied and maintained in culture for up to 7 passages to evaluate their senescence status, so as to avoid studies with CAFs under replicative senescence. Two CAFs had a high degree of senescence and were excluded from the study (Figure 1D).

Primary colon CAFs induce different paracrine pro-migratory effects on cancer cells

A set of co-culture experiments with CAFs and two colon cancer lines were performed to assess the migration-promoting effects of 15 human primary CAFs on colon cancer cells.

Primary CAFs induced a significant increase of co-cultured colon cancer cell migration in a setting in which colon cells are seeded in the upper chamber of the trans-well culture system and fibroblasts in the lower one. After 48 h co-culture, epithelial cells that had reached the
lower surface of the filter were recovered by trypsinization and counted by fluorescence to assess cell migration. Interestingly, we observed significantly large differences of fibroblast-derived paracrine pro-migratory effects on cancer cells in CAFs from different patients. Thus, primary CAFs induced LIM1215 cancer migration between 5 and 140-fold for LIM1215 migration without CAF co-culture stimulation. Based on these data, CAFs were classified into four sub-types as follows: “Low” (L) pro-migratory group, which includes those CAFs unable to increase LIM1215 migration more than 10-fold; “Medium” (M) pro-migratory group, with CAFs showing a 10 to 25-fold increase in colon cell migration; “High” (H) includes CAFs with a 25 to 50-fold induction of colon cell migration; and the “Extra-high” (EH) pro-migratory group contains those CAFs able to enhance colon cell migration more than 50-fold (Figure 2A).

No association was observed, between changes in CAFs’ sub-culture passages/ population doubling number and CAFs derived paracrine pro-migratory effects on cancer cells (Supplementary Figure 1A).

The differences in the fibroblast-derived paracrine pro-migratory effects of CAFs on cancer cells were validated with randomized CAFs from 4 different patients using SW480-ADH cells. Similar results to those previously observed with LIM1215 colon cells were found (Figure 2B). Moreover, a clear correlation between CAF-derived migration induction of LIM1215 and SW480-ADH was observed (Figure 2C).

Finally, the pro-migratory effects on cancer cells of 6 human primary NFs were also analyzed. Although cancer cell migration was induced by NFs regarding migration without
fibroblasts co-culture stimulation, pro-migratory CAF-induced effects were higher than NF effects (Supplementary Figure 1B).

Taken together, these experiments demonstrated that the pro-migratory effects of human cancer-associated fibroblasts on colon cancer cells varied in different patients.

**Enhanced pro-migratory capacity of CAFs correlates with activated fibroblast features**

Various analyses were performed to evaluate whether most pro-migratory CAFs showed activated fibroblast features.

The expression levels of \( \alpha \)-SMA were evaluated in the human primary CAFs. Those CAFs that increased colon cancer cell migration more than 25-fold (“H” and “EH” CAFs) had higher \( \alpha \)-SMA mRNA expression levels than CAFs from “L” or “M” pro-migratory group (Figure 3A).

In parallel, the collagen contractile abilities of primary CAFs were also evaluated as a marker of activated fibroblasts. Therefore, the collagen contraction assay was performed in one selected representative CAF from each migration group. A clear correlation was observed between the induction of collagen gel contraction and pro-migratory effects on cancer cells (Figure 3B).

These data together indicate a clear association between CAFs’ pro-migratory stimulation of colon cancer cells and the activation grade of fibroblasts.

**Most pro-migratory CAFs showed stem cell markers**

The possibility that most pro-migratory CAFs had a stem cell-like phenotype was evaluated.
SOX2, OCT3, KLF4, LIN28 and NANOG genes were used as stem cell markers and their expressions were determined in colon CAFs. Quantitative analysis showed higher expression levels of all analyzed genes in “EH” and “H” pro-migratory groups than in “M” and “L” subtypes. It should be noted that a significant statistical association between OCT3 levels and CAFs’ pro-migratory effects on colon cancer cells was observed (Figure 3C). Moreover, a trend towards statistical significance was observed for SOX2 and LIN28 gene expression and CAFs’ pro-migratory abilities. For the genes KLF4 and NANOG the same trend was observed, but statistical association was not reached, probably due to the small number of samples analyzed and the large difference within CAF groups indicated by standard deviation (Figure 3C).

The pluripotency of different CAFs was also analyzed by osteoblast differentiation experiments in one selected representative CAF from each migration group.

Interestingly, CAFs showed osteoblast differentiation under osteogenic induction medium, assessed by a mineralization quantification assay. However, no association between osteoblast induction and CAF pro-migratory abilities was found (Supplementary Figure 1C).

Together, these data suggest an association between CAFs and stemness characteristics. However, no conclusive evidence was obtained for the association between the degree of CAF stemness and their pro-migratory effect on colon cancer cells.

**Identification of differently expressed genes in CAF pro-migratory subtypes**
To identify differences between fibroblasts with varying pro-migratory impact on colon cancer cells, representative CAFs from each pro-migratory subtype were selected for gene expression profiles by gene microarray analysis.

Four CAF samples, Low (L, id=28), Medium (M, id=32), High (H, id=13) and Extra-High (EH, id=33), and two normal fibroblasts (NF, id=36 and 13) as control samples were analyzed.

To determine the differential expression (DE) profiles of CAF pro-migratory subtypes, the expression value from two NF sample averages was subtracted from four migratory group samples (L, M, H, EH).

Analysis of the samples showed that “L & M” CAF subtypes clustered together and “H & EH” formed another group. Therefore, we considered L & M (LM) as one replicated group and H & EH (HEH) as another (Figure 4A). Significant DE genes were determined by a p-value 0.05 cut-off; and Log2 Fold Change (Log2FC), by a cut-off at 0.5. A detailed gene list with expression values is given in Supplementary Excel file Table S3. These genes were used for further downstream analysis.

The expression of genes in “LM” and “HEH” CAFs was used to generate an initial “CAF signature”, to be used for further analysis including classification of cancer patients and prognostic effects. “Gene-normalized” values with ANOVA statistics (p-value cut-off 0.05) were used to determine this signature. Supplementary Excel file Table S4 contains a detailed list of CAF signature genes.
Functional clustering of CAF pro-migratory subtypes shows involvement of Golgi among other over-represented processes

To better understand the biology behind the DE genes in each LM and HEH group, we explored Gene Ontology Biological Process (GOBP) enrichment analysis with binomial statistics.

Selected enriched GOBP categories are shown in Figure 4B. A full list of enriched terms and statistical details is given in Supplementary Excel file Table S5. Curiously, DE genes in the “protein targeting to Golgi” GOBP module were observed among CAF subtypes. Thus, ‘Golgi’-related processes were significantly over-represented in down-regulated genes of the “LM” group, while the same processes were enriched in up-regulated genes in the “HEH” group. DE genes in the “protein targeting to Golgi” GOBP category in migratory groups are shown in Supplementary Figure 2A.

Notably, DE genes in the “negative regulation of cytokine production” (Supplementary Figure 2B) GOBP category were significantly enriched for down-regulated genes in the “HEH” group. In addition, p53 signal transduction and other biological process related to apoptosis and cell death, such as “signal transduction by p53 class mediator”, “induction of apoptotic genes” or “apoptotic process”, were all also down-regulated in the “HEH” group (Supplementary Figure 2C-1E, respectively).

Finally, different GOBP modules relating to immune response, significantly enriched for down-regulated genes in the “HEH” group, were also indicated, such as “toll-signaling pathways” (Supplementary Figure 2F), “regulation of innate immune response”, “cellular
response to interferon-gamma” and “regulation of type I interferon production” (Figure 4B).

In general terms, gene ontology analysis revealed that “Differentially expressed CAF genes” represent functions or processes of immediate relevance to the tumor microenvironment and include regulators of cytokine production or modulation of the immune system.

“CAF signature” is a prognostic marker of colon cancer patient survival

To assess the prognostic value and clinical relevance of “CAF signatures” in colon cancer, publicly available microarray datasets (a cohort of 232 colon cancer patients from the GEO database) were used. Patients were split into two groups based on the expression difference from median. Using Gitools (Perez-Imaz C and Lopez-Bigas N, 2011), Z-score enrichment was analyzed in the “CAF signature gene module”. This analyzed colon cancer patients who showed significant up- and down-regulation for “CAF signature genes”. Clinical survival information was then correlated with this modular expression to determine (Kaplan-Meier survival analysis) whether “CAF gene signature” had a role in disease prognosis. The detailed gene list used for “CAF signatures” is shown in Supplementary Excel file Table S4 and a heat-map of the top 50 “CAF signature” genes appears in Figure 5A.

The survival analysis showed increased risk factors for Disease-Free Survival (DFS) for patients with high “CAF signature scores” in the data set (HR = 2.2; CI: 1.2-4.1) (Figure 5B). Similarly, a clear trend, almost statistically significant, was observed for Overall Survival (OS) (HR = 1.8; CI: 1.0-3.3) (Figure 5C). Therefore, patients with a similar expression pattern to “CAF signature” showed significantly poorer survival in an independent dataset of colon cancer patients.
Based on these results, the prognostic value of “CAF signature” was analyzed for the different tumor stages. The analysis revealed that “CAF signature” showed prognostic value at advanced stages for DFS and OS, but not at early stages (Figure 5D and 5E and Supplementary Figure 3A and 3B). Therefore, the signature studied behaves as a better predictive variable for survival in advanced stages.

Together, these analyses demonstrate that higher-expression “CAF signature” genes is a marker for poor prognosis. Moreover, the stage sub-group analysis indicates that the “CAF signature” variable is associated with poor prognosis predominantly in advanced-stage patients.

Validation of “CAF signature” genes as prognostic markers of colon cancer patient survival

The de-regulated expression of 7 randomly selected genes from the “CAF signature” was confirmed by RT-qPCR analysis (Supplementary Excel file Table S4 and Figure 6A).

Among these genes, a set of four genes, IGBP3, OAS2, MX1 and ROBO2, was selected for validation as prognostic markers in an independent series of colon cancer patients. The mRNA expression of these genes was analyzed by RT-qPCR in colon cancer-derived samples from 142 patients.

IGFBP3 and ROBO2 showed up-regulated expression levels, while OAS2 and MX1 showed down-regulated levels in “HEH” CAF group. Thus, de-regulated gene expression was considered in patients with higher mRNA expression levels of IGFBP3 and ROBO2 or patients with lower expression levels of OAS2 and MX1.
Individual survival analysis of each gene showed different associations between deregulated expression mRNA levels and patients DFS or OS. However, significant statistical difference was not achieved in all cases (Supplementary Figure 4A and 4B). Nevertheless, the “CAF signature” included the expression of many genes. Thus, the mRNA expression levels of the gene set were combined to study their possible additive effect on patient survival. Interestingly, a clear association was observed between the combination of deregulated gene expressions and colon cancer patients DFS or OS (Figure 6B and 6C).

Together these results support the involvement of “CAF signature” genes in colon cancer patient survival.

**Golgi process as predictor marker of colon cancer patient survival**

As we found over-representation of Golgi-related GOBP (Figure 4B), we determined to analyze this further. Therefore, GOBP Golgi process-related genes were grouped as a single category/module and their suitability for survival prediction and disease prognosis was assessed.

First, all genes associated with Golgi and the related GOBP process (229 Golgi-related gene modules) were used to classify patients into two groups, using Z-score enrichment analysis on expression data: those who showed a higher expression of the Golgi module (including patients with most of their Golgi genes up-regulated) and patients with a lower expression of the Golgi module. Interestingly, Golgi process genes showed a significant predictive value for DFS and OS in colon cancer patients. Therefore, as shown in Supplementary Figure 5A-5C, patient survival is significantly worse in those with a high score of Golgi genes.
DISCUSSION

This study identifies for the first time different CAF subtypes in patients with primary colon carcinomas. Thus, CAFs were functionally classified for their ability to promote migration in cancer cells, their activated fibroblast features and their distinct molecular expression profiles. Interestingly, the association between typical stem-cell markers of CAFs and their pro-migratory effect on tumor cells was also seen. The clinical relevance of these findings is established by the definition of a gene expression signature, derived from the most pro-tumorogenic CAFs, which showed a remarkable prognostic value for the clinical outcome of colon cancer patients.

Growing evidence suggests that primary normal fibroblasts and cancer-associated fibroblasts have distinct gene expression signatures in several tumor types. Thus, in breast cancer, significant differences between CAFs and normal counterparts or CAFs derived from different clinical subtypes have been described (21-23). In non-small cell lung cancer, genes relating to the TGFβ signaling pathway are differently expressed by NFs and CAFs (24). The transcripts expressed by Tag profiling in CAFs, normal human prostate fibroblast and fetal cells were also compared in one prostate cancer patient (25). Identified clusters of CAF gene signatures characterized genetically unstable and stable Oral Squamous Cell Carcinoma (OSCC) in relation to each other and to fibroblasts from normal oral mucosa (26). Furthermore, distinct expressions of regulatory miRNA in CAFs derived from CAFs vs paired NFs were determined in endometrial and bladder cancer (27, 28).

Despite the above-mentioned findings, CAFs from primary colon cancer and paired NFs have not yet been fully studied. The growth-promoting effects on colon cancer cells in vitro...
of fibroblast cultures from metastatic colon cancer in liver or from liver away from metastatic lesions and skin from three patients were determined, as was the molecular profile expression of CAFs and skin fibroblasts (29). Similar efforts were made to identify protein expression patterns in CAFs and NFs from colon cancer patients (30). These two studies described differences between CAFs and NFs, but no clinical study to back up their findings was undertaken.

In this scenario, this is the first study to demonstrate the pro-migratory impact of colon CAFs, from different patients, on cancer cells and to show that they have greater impact than NFs do. The migration experiments with two cell lines suggest that CAF pro-migratory effects are not restricted to a particular cell line. The phenotypical characterization of activated fibroblast markers, studied by α-SMA staining and collagen contractibility assays, suggests a link between the different activation level of CAFs and their potential to enhance tumorogenesis. In general terms, CAFs collectively share the same activation state, but their expression of activation markers, such as α-SMA, FSP1, FAP or others, may differ (31). Supporting our data, a recent study demonstrated that the silencing of the FAP gene, another classic marker of fibroblast activation, in CAFs drives the reduction of tumor growth in vivo and of tumorogenesis in ovarian cancer (32). In this same study, FAP silencing and its effects are mediated in some way by down-regulation of the stem cell markers in CAFs. Thus, it cannot be ruled out that the acquisition of the stem cell characteristics of CAFs could determine their pro-tumorogenic abilities. Supporting this idea, preliminary findings of the present study showed associations between CAF stem cell markers and their pro-migratory abilities in colon cells. Moreover, experiments showed osteoblast cell differentiation as a CAF stemness characteristic. However, no association was observed
between the degree of CAF differentiation and CAFs’ pro-migratory effect on cancer cells. The potential involvement of stemness in the CAF-dependent migration of colon cells needs to be studied by direct approaches. Such studies should particularly explore the stemness characterization of CAFs. Future studies should also address the possibility that CAF pro-migratory abilities could be linked to a non-differentiated stemness phenotype.

CAFs are heterogeneous populations and their relative composition differs greatly between tumors, within a given tumor type and even in individual tumors (33). This wide heterogeneity in CAFs may be explained by their possibly miscellaneous origin. Indeed, CAFs are variously reported to derive from resident fibroblast, bone marrow-derived progenitor cells, endothelial or cancer cells through endothelial/epithelial-mesenchymal transition, smooth muscle cells, pericytes, adipocytes or inflammatory cells (34). The effect of different CAF sub-populations on different CAF pro-migratory abilities should be further analyzed by expanded analysis of cell origin traceability.

As expected, gene ontology analysis revealed that CAF gene expression represents functions or processes of immediate relevance to the tumor microenvironment, including regulators of cytokine production or modulation of the immune system. The down-regulation of genes relating to "negative regulation of cytokine production" was a particular feature in most pro-tumorogenic CAF groups. This finding suggests that genes that negatively regulate cytokine production could be switched off and hence more cytokines may be produced by this CAF population subtype. Down-regulation of immune response pathways for this CAF sub-population could be associated with induced immune response suppression by primary tumor. Finally, p53 signal transduction and other biological processes relating to apoptosis
and cell death were all also down-regulated in this CAF group. The activation state of these cells may explain the down-regulation of these pathways.

Surprisingly, differences in pathways relating to “protein targeting to Golgi” between CAF subtypes were observed. In line with the data presented, recent studies have demonstrated that over-expression of Golgi proteins, such as Golgi phosphoprotein 3 in cancer cells, correlated with their proliferation and tumorigenicity in vitro (35-39). In addition, descriptions of the molecular network, connecting the Golgi to other organelles, demonstrate the essential roles of the Golgi in different cellular activities, indicating their possible role in prostate tumors (40). In inflammatory bowel disease, altered glycosylation, which occurs in the Golgi apparatus, may be a factor in colon cancer development (41).

A few studies identified a prognostic “CAF signature”, compared to NFs, that was associated with poor patient prognosis. In non-small cell lung cancer, analysis of CAFs and NFs identified a subset of differentially expressed genes with prognostic value in multiple independently published expression datasets of primary non-small cell lung cancer (24). In oral squamous cell carcinoma, the gene expression signature of fibroblasts from genetically unstable OSCC was associated with poor clinical outcome in an independent microarray database of head and neck cancer patients (26). On microdissecting total stroma cells from Barret esophagus tumors, a gene signature discriminates pre-invasive from invasive disease in digestive cancers, including colon cancer. However, the prognosis value of this stromal signature was not confirmed in any tumor (42). The data reported here imply the clinical relevance of the specific “CAF signature” in colon cancer. Supporting this finding, an association between the expression levels of a four-gene set, included in the “CAF
signature”, and prognostic value in an independent series of 142 colon cancer patients was observed. Due to a lack of available public stromal/epithelial ratio data, data extracted from public datasets were applied to whole tumor tissue, contrasting with the identified “CAF signature”. Better characterization of the impact of epithelial contamination is also required. Preliminary studies with our CAFs and whole tumor sample counterparts were not sufficient to reach any conclusive results (data not shown). A large array analysis with many samples would be required, and although this issue could be a limitation of the study, the “in silico” analysis, in an independent dataset of colon cancer patients, confirmed the prognostic value of most pro-tumorogenic “CAF signatures”. Similar procedures were developed in the few studies in which prognosis was associated with a prognostic “CAF signature”, when compared to NFs (24, 26). It should be noted that the signature studied behaves as a better predictive variable for patient outcome in patients with advanced-stage tumors. This confirms the view that development of new therapies, targeting microenvironment components, would help to improve the medical treatment of those patients usually with worse outcome. Finally, the involvement of the Golgi process suggested by gene ontology analysis is also clinically supported by the specific predictive value of Golgi process genes for clinical outcome in colon cancer patients. Better characterization of Golgi effects in colon tumors will be needed.

The findings of this study demonstrate the important role of CAF gene expression changes in colon cancer patients. These changes involve enhancement of colon cell migration. The findings contribute to the identification of colon cancer cell prognostic markers. They emphasize the ongoing efforts to discover the real involvement of CAF changes in cancer
progression and encourage the search for new stromal treatment targets that would combine effectively with current systemic therapies.
ACKNOWLEDGEMENTS

M. Eaude assisted with the English text. Members of the FB laboratory contributed with constructive criticism throughout this project.

GRANT SUPPORT

This study was supported by the Fundación Científica AECC, PI12/02037, SAF2010-20750, CM-S2010/BMD-2344, RD12/0036/0041 and the Fundación Banco Santander. Antonio García de Herreros’ laboratory was supported by RD12/0036/0005. Cristina Peña is a recipient of a Miguel Servet Contract from the Instituto de Salud Carlos III (CP09/00294); and Vanesa García, of a contract from AECC.
FIGURE LEGENDS

**Figure 1. Establishment and characterization of primary CAFs from colorectal cancer patients.** Schedule of primary CAF establishment from fresh tissue of primary tumors from colorectal cancer patients (A). Although at different levels all CAFs showed α-SMA expression. In the images, examples of a low and a high α-SMA-expressing CAF are given (BI and BII, respectively). The absence of epithelial cells in the established primary cultures was confirmed by PanCK and Vimentin immunostaining. LIM1215 colon cells were used as positive control for PanCK immunostaining (C). Senescence-Associated β-galactosidase staining was used to evaluate the degree of senescence of primary CAF cultures (DI showed a negative CAF culture and DII, a positive CAF culture for SA β-galactosidase staining).

**Figure 2. Differences of human primary CAF-derived paracrine pro-migratory effects on colon cancer cells.** Primary CAFs were classified for their ability to stimulate LIM1215 colon cell migration as “Low”, “Medium”, “High” and “Extra-high” migration groups. The number in the X-axis denotes internal colon patient IDs (A). Pro-migratory enhancement of colon cell migration was also validated in SW480-ADH colon cells with 4 randomized CAFs (B). Migratory stimulation of LIM1215 and SW480-ADH cells, depending on co-cultured CAFs, showed statistical correlation (C).

**Figure 3. Colon CAF-derived paracrine pro-migratory effects are associated with activated fibroblast features and stemness markers.** Most pro-migratory CAFs (“H” and “EH”) showed higher α-SMA mRNA expression levels (A). Association between primary colon CAF pro-migratory effects and collagen gel contraction (BI). BII showed an example of contraction measurement: black circles and upper pictures are CAFs embedded in...
collagen gel at 0 hours. Lower pictures show previous measurements (black circles) and new measurements (white circles) after 24h (both pictures are shown by replicates) (BII). Generally, an increase in stemness markers, measured by RT-PCR, was seen in most pro-migratory CAFs. Remarkably, there was significant statistical association with OCT3 expression levels and a trend was also observed with SOX2 and LIN28 expression levels (C).

**Figure 4. CAFs clustering and functional enrichment analysis of differentially expressed genes.** False color heat-map of the distances between arrays (CAF samples) (A). The color scale was chosen to cover the range of distances encountered in the dataset. Patterns in this plot indicate clustering of the arrays for intended biological similarities. With expression values of each of the 4 samples, distant similarity matrices were calculated by the program arrayQualityMetrics (see Methodology for description). Enrichment of selected Gene Ontology (GO) Biological Process (BP) categories under different experimental conditions is seen (B). Columns give different comparison conditions between two experimental conditions. With differentially expressed genes in these conditions, functional over-representation is shown in rows on the color-coded heat-map. Color represents corrected (FDR) p-values. The reddish color indicates a more significant p-value, whereas yellow indicates a less significant one and gray indicates not significant.

**Figure 5. CAF gene signature and its capacity to identify prognostic outcome.** The top 25 up-regulated and top 25 down-regulated CAF signature genes in color-coded heat-map (A). Survivability of colon patients expressing higher and lower expression of CAF-signature genes (patients grouped on the basis of the Z-score of the CAF signature gene
module on median centered expression colon dataset – see Methodology for details). The Hazard Ratio (HR) was based on the Cox model (B, C). Similarly, the ability to predict the stage-prognostic capacity of CAF signature was tested with the Kaplan-Meier plot on colon patients’ microarray expression data. Patient samples were grouped in stages I-II and III-IV (D, E), based on information provided by authors of this public data (see Methodology for dataset reference). Survivability of patients expressing or non-expressing CAF signature was tested as described above.

**Figure 6:** Validation of de-regulated genes and prognostic value of “CAF signature” by RT-qPCR. The mRNA expression of 7 randomly selected genes from the “CAF signature” was analyzed by RT-qPCR. Six of the seven showed statistical differences for mRNA expression between the “Low plus Medium” and “High plus Extra-high” migration groups (A). A clear association was observed between the combination of four gene expression data, including IGFBP3, OAS2, MX1 and ROBO2, and colon cancer patient DFS (B) or OS (C).
REFERENCES


FIGURE 1

A

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B

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FIG. 6

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B

Cumulative survival

- $p < 0.001$
- HR (1 vs 0) = 2.4 (0.3-20.9)
- HR (2 vs 0) = 3.4 (0.4-26.3)
- HR (3 vs 0) = 5.8 (0.7-52.4)
- HR (4 vs 0) = 85.6 (4.4-1672.9)

C

Cumulative survival

- $p < 0.001$
- HR (1 vs 0) = 8.1 (1.1-61.2)
- HR (2 vs 0) = 9.4 (1.3-69.5)
- HR (3 vs 0) = 14 (1.7-112.5)
- HR (4 vs 0) = 27.5 (1.7-450.1)
FUNCTIONAL HETEROGENEITY OF CANCER-ASSOCIATED FIBROBLASTS FROM HUMAN COLON TUMORS SHOWS SPECIFIC PROGNOSTIC GENE EXPRESSION SIGNATURE


*Clin Cancer Res* Published OnlineFirst September 19, 2013.

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