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Colorectal Cancer-Associated Fibroblasts are Genotypically Distinct

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Abstract

Cells in the stromal microenvironment facilitate colorectal cancer (CRC) progression and "co-evolve" with the epithelial cancer cells. Genetic and epigenetic differences between normal colorectal mucosa fibroblasts (NF) and carcinoma-associated fibroblasts (CAF) are not known. The aim of this study is to identify differentially expressed genes and promoter methylation between NF and CAF in human CRC. RNA and DNA were extracted from cultured NF and CAF from CRC resections. Genome-wide gene expression and methylation analyses were performed using the Illumina Human HT-12 v4.0 Expression and Illumina Human Methylation 27 BeadChips. Gene expression values between NF and CAF were compared and correlated with methylation patterns. Data was analyzed using Partek Genomics Suite using one-way ANOVA and $p<0.05$ as significant. Ingenuity iReport™ was performed to identify potential differences in biological functions and pathways between the NF and CAF. Paired methylation and gene expression analyses from 11 NF and 10 CAF colorectal samples are reported. Unsupervised analysis of differentially expressed genes using iReport™ identified "Top Diseases" as "Cancer" and "Colorectal Cancer". Previous genome wide studies have focused on the cancer cells. We have identified differentially expressed genes and differentially methylated promoter regions that are CAF-specific in CRC.

Keywords

carcinoma-associated fibroblasts; colorectal cancer; gene expression; normal fibroblasts; microarray; promoter methylation

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CONFLICTS OF INTEREST
The authors have no conflicts of interest.

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Conception and design (CC, MRH JRC, TW), analysis and interpretation (CC, JRC, AM), data collection (CC, JRC, ME, FJB, AM) writing the article (CC, AM), critical revision of the article (n/a) and obtaining funding (CC, MRH).

INTRODUCTION

Gene expression profilings for colorectal cancer (CRC) samples have been performed using RNA extracted from whole tissue tumor samples. Since the tumor microenvironment is critical to the biological behavior of the cancer, elucidating the role of carcinoma-associated fibroblasts (CAF), the most abundant cell-type in the stroma, is crucial to understanding the pathogenesis of CRC [1]. A stroma-derived gene signature has been shown to correlate with prognosis, suggesting that tumor stroma contributes to the progression and metastatic potential of CRC [2–4].

Experimental data support the contention that fibroblasts associated with the normal colonic epithelium (NF) are phenotypically different from CAFs [5, 6]. Stable gene expression changes in CAF may be due to epigenetic changes [7] versus somatic mutations [8, 9]. It is now known that somatic mutations in the DNA sequence of CAF are rarely, if ever, encountered [10, 11], and thus, the acquisition of tumor-promoting activities by CAF, in part, are due to epigenetic alterations in the DNA [7, 12]. The most studied epigenetic modification is DNA methylation, which occurs on CpG islands within the gene promoter region. Genes are downregulated when promoter regions are heavily methylated, a process that entails the methyl donor S-adenosylmethionine transferring a methyl group to the 5’ carbon of cytosine.

The purpose of this study is to determine differences in the gene expression of resident fibroblasts in the normal colon mucosa (NF) versus CAF in human colorectal cancer and to determine which differentially expressed genes may be regulated by promoter methylation.

MATERIAL AND METHODS

Fibroblast isolation and culture

Under an IRB-approved discarded tissue protocol, human colon-derived fibroblasts were isolated from freshly resected operative specimens at the University of Texas Medical Branch, Galveston, TX. Surgical pathologists excised approximately 500 mm^3 of tissue from grossly recognizable tumor and/or adjacent normal mucosa. In some cases, the normal mucosa from colectomies for diverticular disease or large adenomas was obtained for culture. Fibroblasts were derived from 10 normal colonic mucosa and 11 adenocarcinomas as previously described [13]. Primary CAF and NF cultures were routinely maintained in DMEM and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Relevant clinical and histopathologic information was extracted via retrospective chart reviews under a second IRB protocol (Table 1).

Microarray analyses

Total RNA was extracted using RNAqueous (Ambion) from NF and CAF early passage (P2-5) cultures. The purity and concentration of the RNA samples were determined using Agilent 2100 Bioanalyzer and NanoDrop ND-1000, respectively. Samples of cRNA were hybridized to Illumina Human HT-12 v4.0 Expression BeadChip, which covered the whole genome with over 47,000 probes. Genomic DNA was extracted from parallel cell cultures with lysis buffer (0.6% SDS, 10 mM EDTA, 10 mM Tris HCl, pH 7.5 and 100 μg/ml

RNase-A). DNA underwent phenol and chloroform extraction, was precipitated with ethanol, and was rehydrated with sterile H_20. Genomic DNA from each sample was modified by sodium bisulfite conversion and enzymatic fragmentation, and then hybridized to the Human Methylation27 BeadChip which interrogated 27,578 CpG loci, corresponding to 14,495 genes. Two probes for each CpG site are present, one corresponding to a methylated CpG locus and the other to the nonmethylated locus. Allele-specific primers are annealled and extended with DNP- and biotin-labeled ddNTPs. The array is fluorescently stained, scanned, and the intensities of the nonmethylated and methylated bead types are measured. The BeadStudio Software generated a DNA methylation level (β value) for each CpG site, which is determined by dividing the signal intensity for the methylated CpG by the sum of both the methylated and unmethylated CpGs. Beta values with a value of "0" indicate no methylation and "1" indicate a methylated promoter region. Quality control and interarray normalization was conducted according to manufacturer standards for each array. One sample failed the gene expression array quality control and another sample failed the methylation array; both were removed from the study, leaving a final N=11 NF and N=10 CAF samples for analyses.

Statistical analysis

Microarray data for both chips were analyzed using Partek Genomics Suite (Partek, Inc; St. Louis, MO). Data quality was assessed via PCA and the application of Partek's quality control workflow. Differentially expressed candidate genes and differential β-values between comparison groups were identified by applying ANOVA, $p<0.05$. Thus, two filters, each with p- values <0.05, were applied to the final combined dataset, in order to decrease the false discovery rate. Ingenuity iReport™ for the microarray analysis (Qiagen, Valencia, CA) was performed to identify potential differences in biological functions and pathways between the NF and CAF.

All data used in this study are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE XX, and GSE YY.

RESULTS

Establishment of primary fibroblast cell lines

The fibroblast cell lines are derived from either male or female patients and used without regard to sex. The purity of the fibroblast cultures were verified as previously described using anti-vimentin (V6630 1:40, Sigma), anti-α-smooth muscle actin (αSMA) (A5228; 1:200, Sigma), and anti-pancytokeratin antibodies (AE1/3 1:200 Santa Cruz) (data not shown) [13]. The antibody to CD31 was used in several cultures to demonstrate lack of endothelial cell contamination (data not shown). The clinico-pathologic data from the 11 NF and 10 CAF samples are shown in Table 1. Then NF was derived from the normal margin of colectomies for either cancer or benign lesions such as diverticulitis; 2 samples were derived from tubovillous adenomas. The CAFs were obtained from mainly Stage II and III operable colorectal cancers. One sample was from a patient who was found to have distant metastatic disease intraoperatively.

Differential gene expression in CAF vs. NF

Unsupervised cluster analysis (Partek Genomics Suite) of gene expression profiles generated using the Human HT-12 v4.0 Expression BeadChip array comparing NF and CAF identified 2,472 differentially expressed genes with p-values <0.05. Compared to NF, CAF overexpressed 1,168 (47.2%) gene transcripts. In contrast, NF over-expressed 1,304 genes (52.8%) compared to CAF. If the p-value was set at p<0.001, only 84 genes were significant between the two groups (Fig. 1). Fifty genes (60%) were over-expressed in NF relative to CAF, whereas 34 genes (40%) were over-expressed in CAF relative to NF.

Ingenuity pathway analyses of differentially expressed genes

We performed an unbiased analysis using iReport™, which offers pathway, process and disease associations without the input of key words. Unlike the Ingenuity Pathway Analysis which requires input of filters and key words, Ingenuity iReport™ identified “Cancer”, “Metastatic Colorectal Cancer” and “Colorectal Cancer” as “Top Diseases”. For example, tumor suppressor gene Deleted in Colon Cancer was decreased in the CAF compared to NF and microRNAs specific to colon cancer (MIR653 and MIR601) were upregulated in CAF. Cell Migration and Invasion were identified as “Top Processes”. Finally, “Top Pathways” identified the Wnt/βcatenin signaling pathway, and the FXR/RXR nuclear receptors which are conditional (ligand-activated) transcription factors that either have been implicated in or associated with colon cancer progression [14–16].

Specific genes upregulated in CAF which may promote tumor cell invasion by increasing cell adhesion and/or migration and invasion include: COL4A6, ITGB1BP1, CEACAM20, NCAM1, NEDD9, CD34, EXOC2, SMAGP, GLT25D2, TIAM2, LIM2, RAB11FIP1, and PRKD2. Genes that may enhance CAF-mediated cell-cell interaction signaling (PCDHGB6, PCDHGA9, IL12RB1, CD83, IL1RAP, IL18RAP, TAC1, LYVE-1), and promote extracellular region degradation (HYAL3, ADAMTS1, ADAMTS7, MMP16, GPR65, PCSK6, USP22) were over-expressed in CAF compared to NF. Other differentially overexpressed genes in CAFs include growth factors (HGF, CDNF, and CSH1), transcriptional regulators (IRF5, KLF5, ELK4, SSX4B, and ATF3), cell survival/ proliferation-associated genes (AKT1, CDKL2, CNNM1, CCNE1, NOL3, GRB2, and PRAMEF19), members of the Wnt signaling pathway (SNAI3), and other intracellular signaling cascades (PLCB3, ERBB3, and BMX). Several genes encode secreted proteins (NRG4, TNF9, PCSK6, CMTM4, BOLA2, and TNSF8) that may also contribute to tumor progression (see GSEXX).

Differential methylation between CAF vs. NF

A total of 1,772 differential methylation of cytosine residues at CpG dinucleotides were identified between NF and CAF: 1,057 (60%) were hypomethylated and 715 (40%) were hypermethylated in CAF compared to NF. To correlate differentially expressed transcripts, which may be regulated epigenetically by promoter methylation, we matched the gene expression data with differentially methylated CpG loci between NF and CAF. Compared to NF, CAF overexpressed 26 genes corresponding with promoter hypomethylation, and CAF cells had decreased expression in 33 genes corresponding with promoter hypermethylation (Table 2). Among the 26 overexpressed genes in CAF, 6 (23%) genes are located on

chromosome 6, and among the 33 hypermethylated or under-expressed genes, 4 (12%) were located on chromosome 9.

DISCUSSION

We identified differentially expressed genes in CAF primarily cultured from human colorectal cancer specimens compared to NF from normal/non-malignant colorectal tissue obtained from surgical resections. Although primary cultures from tissue explants may be biased by clonal selection [10], it assures higher purity of the tissue population (fibroblasts) studied. Laser capture microdissection will allow for isolating stromal tissue components *in situ*, but introduces confounders, such as the inclusion of other cells from the stroma, e.g., endothelial cells and immune cells.

Importantly, the tumor-promoting phenotype persists when early passage CAF are cultured in absence of carcinoma cells [17, 18], suggesting hereditable changes in CAF. However, genetic mutations in CAF have been found to be largely the result of experimental artifact [11]. Both DNA methylation and miRNA-mediated epigenetic changes have been identified in CAF [7, 12], but our understanding of genotypic differences between colorectal CAF versus NF are limited and require further study.

Chang et al. [19] cultured fibroblasts in 10% fetal bovine serum from ten different anatomical sites and identified 512 genes, which he named “the fibroblast core serum response (CSR) genes”. The CSR genes resemble a wound response signature. The authors showed that the CSR signature was present in cancer tissues from breast, lung, prostate, gastric and hepatocellular origin, but not in corresponding normal tissue [19]. However, Troester and colleagues [20] showed that the CSR signature was prominent in “normal” breast tissue from the margin of breast cancers, but not in tissue from reduction mammoplasties. Thus, if applied colorectal cancers, the specificity of a CSR gene set may not accurately distinguish NF and CAF. Furthermore, the CSR gene set is not specific for CRC, since multiple solid tumors were found to have the CSR gene signature. Analysis of our list of differentially expressed genes between CAF and NF using Ingenuity iReport™ demonstrated that CAF are involved in cell migration, invasion and Wnt/β-catenin signaling, as may be predicted based on the known functions of fibroblasts, similar to the functions of the genes on the CSR list. Additionally, unsupervised analyses of our gene list by iReport™ identified “Top Diseases” to be “Cancer”, and specifically “Colorectal Cancer”/“Metastatic Colorectal Cancer”. Importantly, our analysis identified differentially expressed genes that demonstrate both tissue specificity (colorectum) and also distinguishes between NF versus CAF.

CONCLUSIONS

The study of gene expression and its regulation in colorectal CAF is critical to the understanding of how the cancer microenvironment facilitates carcinogenesis and promotes cancer metastasis. Using the Illumina Human Methylation27 BeadChip, we further identified CAF genes that may be regulated by promoter regulation. One limitation of our study is that other mechanisms of epigenetic regulation of gene expression were not studied

(for example, histone modification). Additionally, followup studies will be needed for independent technical validations of gene expression and the methylation of CpG sites, and verification of the gene expression-promoter methylation relationships in an independent set of colorectal NF and CAF. Future studies to identify specific epigenetic step-wise changes in the transition from NF to CAF in colorectal cancer are needed to develop therapies that target CAF in the cancer microenvironment [21].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

CRC	colorectal cancer
CAF	carcinoma-associated fibroblasts

REFERENCES

1. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer. 2008; 123(10):2229–2238. [PubMed: 18777559]
2. Huijbers A, Tollenaar RA, v Pelt GW, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013; 24(1):179–185. [PubMed: 22865778]
3. Ngan CY, Yamamoto H, Seshimo I, et al. Quantitative evaluation of vimentin expression in tumour stroma of colorectal cancer. Br J Cancer. 2007; 96(6):986–992. [PubMed: 17325702]
4. Wikberg ML, Edin S, Lundberg IV, et al. High intratumoral expression of fibroblast activation protein (FAP) in colon cancer is associated with poorer patient prognosis. Tumour Biol. 2013; 34(2):1013–1020. [PubMed: 23328994]
5. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res. 1999; 59(19):5002–5011. [PubMed: 10519415]
6. Kuperwasser C, Chavarria T, Wu M, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A. 2004; 101(14):4966–4971. [PubMed: 15051869]
7. Hu M, Yao J, Cai L, et al. Distinct epigenetic changes in the stromal cells of breast cancers. Nat Genet. 2005; 37(8):899–905. [PubMed: 16007089]
8. Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou XP, Eng C. Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. Nat Genet. 2002; 32(3): 355–357. [PubMed: 12379854]
9. Weber F, Shen L, Fukino K, et al. Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. Am J Hum Genet. 2006; 78(6):961–972. [PubMed: 16685647]

10. Jiang L, Gonda TA, Gamble MV, et al. Global hypomethylation of genomic DNA in cancer-associated myofibroblasts. Cancer Res. 2008; 68(23):9900–9908. [PubMed: 19047171]
11. Campbell I, Polyak K, Haviv I. Clonal mutations in the cancer-associated fibroblasts: the case against genetic coevolution. Cancer Res. 2009; 69(17):6765–6769. [PubMed: 19706773]
12. Mitra AK, Zillhardt M, Hua Y, et al. MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. Cancer Discov. 2012; 2(12):1100–1108. [PubMed: 23171795]
13. Chao C, Tallman ML, Ives KL, Townsend CM Jr, Hellmich MR. Gastrointestinal hormone receptors in primary human colorectal carcinomas. J Surg Res. 2005; 129(2):313–321. [PubMed: 16051276]
14. Lax S, Schauer G, Prein K, et al. Expression of the nuclear bile acid receptor/farnesoid X receptor is reduced in human colon carcinoma compared to nonneoplastic mucosa independent from site and may be associated with adverse prognosis. Int J Cancer. 2012; 130(10):2232–2239. [PubMed: 21780109]
15. Modica S, Murzilli S, Salvatore L, Schmidt DR, Moschetta A. Nuclear bile acid receptor FXR protects against intestinal tumorigenesis. Cancer Res. 2008; 68(23):9589–9594. [PubMed: 19047134]
16. Jacobs ET, Martinez ME, Campbell PT, et al. Genetic variation in the retinoid X receptor and calcium-sensing receptor and risk of colorectal cancer in the Colon Cancer Family Registry. Carcinogenesis. 2010; 31(8):1412–1416. [PubMed: 20558521]
17. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121(3):335–348. [PubMed: 15882617]
18. Ishii G, Hashimoto H, Asada K, et al. Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: a novel character of tumor educated fibroblasts. Int J Oncol. 2010; 37(2):317–325. [PubMed: 20596659]
19. Chang HY, Sneddon JB, Alizadeh AA, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: Similarities between tumors and wounds. Plos Biol. 2004; 2(2):206–214.
20. Troester MA, Lee MH, Carter M, et al. Activation of host wound responses in breast cancer microenvironment. Clin Cancer Res. 2009; 15(22):7020–7028. [PubMed: 19887484]
21. Takebe N, Ivy P, Timmer W, Khan N, Schulz T, Harris PJ. Review of cancer - associated fibroblasts and therapies that interfere with their activity. Tumor Microenvironment and Therapy. 2013; 1:19–36.

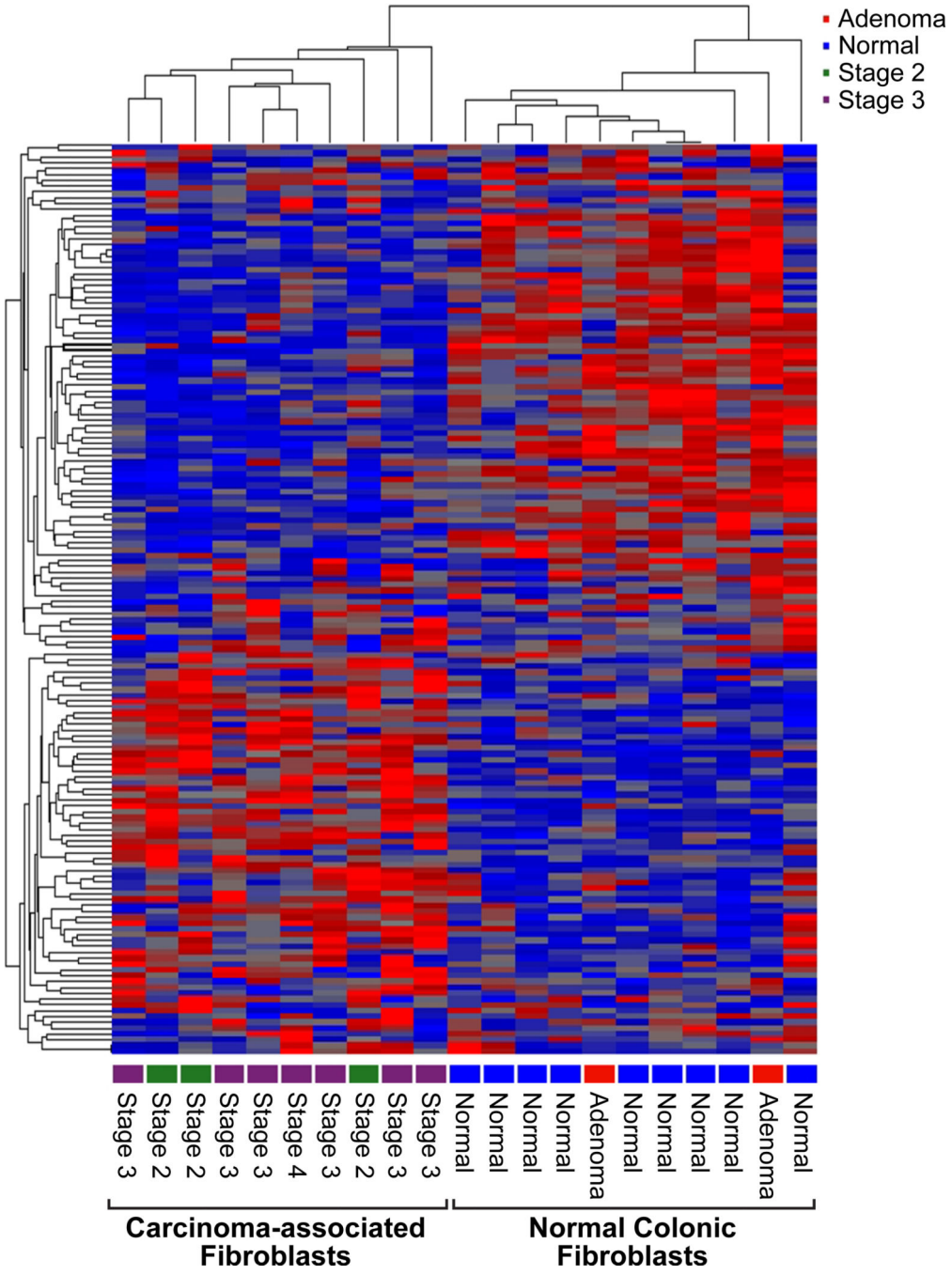

Figure 1.

Heat Map showing differential gene expression of colorectal derived carcinoma-associated fibroblasts (CAF) versus normal mucosal fibroblasts (NF), ANOVA, $p<0.001$.

Table 1

Patient clinico-pathologic data of cultured fibroblasts.

Sample	CRC Stage vs. Non-Malignant Disease	Age	Sex	Pathology
CAF-1	Stage 2 CRC	68	f	Moderately differentiated
CAF-2	Stage 3 CRC	59	f	Poorly differentiated
CAF-3	Stage 4 CRC	62	f	Moderately differentiated
CAF-4	Stage 3 CRC	54	f	Moderately differentiated
CAF-5	Stage 2 CRC	52	f	Poorly differentiated
CAF-6	Stage 3 CRC	56	m	Moderately differentiated
CAF-7	Stage 3 CRC	77	m	Moderately differentiated
CAF-8	Stage 2 CRC	55	m	Moderately differentiated
CAF-9	Stage 3 CRC	76	m	Well differentiated
CAF-10	Stage 3 CRC	67	m	Moderately differentiated
NF-1	Normal	61	f	Normal margin: diverticulitis
NF-2	Normal	38	f	Normal margin: diverticulitis
NF-3	Normal	46	m	Normal margin: diverticulitis
NF-4	Normal	75	m	Vascular congestion
NF-5	Normal	77	f	Normal margin: diverticulitis
NF-6	Normal	74	m	Normal margin: cancer
NF-7	Normal	46	m	Normal margin: cancer
NF-8	Normal	52	f	Normal margin: in situ cancer
NF-9	Tubovillous Adenoma	68	f	Normal Margin: Adenoma
NF-10	Normal	60	f	Normal margin: cancer
NF-11	Tubovillous Adenoma	59	m	Normal Margin: Adenoma

Abbreviations: CAF: carcinoma-associated fibroblasts, NF: normal fibroblasts, CRC: colorectal carcinoma; f: female, m: male.

Table 2

Genes regulated by promoter regulation: A. 26 genes overexpressed in CAF compared to NF.

Function	Probeset	Gene name	Gene Symbol	Fold Change	P value
Metabolism/Transport	cg13077930	Albumin	ALB	1.71E+18	0.000272652
	cg26984624	Ankrin 1, erythrocytic	ANK1	3.48E+07	0.00736581
	cg20857947	Arginisuccinate lyase	ASL	121184	0.027152
	cg22063653	2,3 bisphosphoglycerate mutase	BPGM	28981.8	0.0218035
	cg09425611	Carboxylesterase 1	CES1	431.142	0.0358082
	cg00901704	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	SLC25A19	1.83E+27	0.0170674
	cg08724474	Solute carrier family 36 (proton/amino acid symporter), member 1	SLC36A1	1.77E+36	0.0304509
	cg14265670	Uridine phosphorylase 1	UPP1	2.91E+07	0.0365465
	cg18412613	YKT6-v-SNARE homolog	YKT6	478304	0.0461634
	cg14493899	Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	TAP2	2.17E+14	0.0232357
Adhesion/Signaling	cg01288598	CD83 molecule	CD83	68474.7	0.0271523
	cg05649009	Cholinergic receptor, nicotinic, alpha1 (muscle)	CHRNA1	4.72E+27	0.049423
	cg11552293	Collagen, type IV, alpha 6	COL4A6	1067.49	0.030782
	cg11393453	GRB2 associated regulator of MAPK1	FAM59A	1.61E+10	0.0329179
	cg13060405	Myosin XVIIIA	MYO18A	1.43E+27	0.0304239
	cg25187533	Pregnancy specific beta-1-glycoprotein 2	PSG2	1.13E+32	0.000976575
	cg00950418	SRSF protein kinase 2	SRPK2	1.41E+10	0.0240908
Cell cycle	cg25522312	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	3.81E+07	0.0084277
	cg08062469	Sperm associated antigen 5	SPAG5	4.13E+09	0.0369162
	cg19738333	Receptor accessory protein 4	REEP4	6.08E+12	0.018247
Transcription	cg20150743	Zinc finger protein 3	ZNF3	9578.43	0.0128924
Autophagy	cg10732834	Ectopic P-granules autophagy protein 5 homolog (C. elegans)	KIAA1632	5.03E+33	0.0311191
Unknown	cg22010317	chromosome X open reading frame 36	Cxorf36	43048.4	0.0126714
	cg17178888		FLJ14166	7.39E+07	0.0392013
	cg02890926	Transmembrane protein 53	TMEM53	1.40E+06	0.02627
	cg25861458		FL23356	130539	0.00114061

B. 33 Genes under-expressed in CAF compared to NF

Function	Probeset	Gene name	Gene Symbol	Fold Change	P value
Signaling	cg22930187	Artemin	ARTN	−4008.21	0.0195053
	cg04929736	Clusterin	CLU	−1.21E+14	0.0126985
	cg03278643	Ellis van Creveld syndrome 2	EVC2	−9.16E+06	0.022855
	cg24888049	Feline sarcoma oncogene	FES	−1.31E+29	0.005809
	cg18977436	Fibroblast growth factor 14	FGF14	−68717.7	0.025556
	cg24222324	Tumor necrosis factor superfamily, member 11	TNFSF11	−2227.31	0.008463
	cg11072113	von Willebrand factor C and EGF domains	VWCE	−1.21E+18	0.049167
Cell-cell; cytoskeletal	cg02512860	Claudin 15	CLDN15	−37932.3	0.025022
	cg06799664	Dynein, axonemal, heavy chain like 1	DNAHL1	−6381.26	0.044435
	cg07126559	Sarcoglycan, gamma (35kDa dystrophinassociated glycoprotein)	SGCG	−6.45E+29	0.03913
Enzyme	cg22333888	ADAM metallopeptidase domain 33	ADAM33	−1.33E+29	0.0146697
	cg23026995	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	XPNPEP2	−2.29E+23	0.030756
Transcription factors	cg19556572	AT-hook transcription factor	AKNA	−5.29E+35	0.007186
	cg10003443	forkhead box A2	FOXA2	−7451.05	0.012987
	cg08789630	K(lysine) acetyltransferase 6B	MYST4	−1.74E+07	0.029483
	cg16722536	T-box 1	TBX1	−17684	0.020172
	cg12788467	HNF1 homeobox B	TCF2	−2542.91	0.037723
	cg19352038	Paired box 3	PAX3	−27179.6	0.013469
	cg07403255	Paired box 8	PAX8	−6.64E+06	0.022095
Apoptosis	cg06493930	Growth arrest-specific 2	GAS2	−699.226	0.021412
	cg05924583	Tumor protein p73	TP73	−211.453	0.027257
Transport	cg27461196	FXYD domain containing ion transport regulator 1	FXYD1	−1.94E+21	0.048906
	cg04502814	Selenoprotein P, plasma, 1	SEPP1	−1.24E+13	0.033794
	cg16509045	transient receptor potential cation channel, subfamily M, member 6	TRPM6	−600.168	0.030977
Metabolism	cg10432859	UDP glucuronosyltransferase 1 family, polypeptide A7	UGT1A7	−11322.6	0.017452
Unknown	cg01813965	Coiled-coil domain containing 135	C16orf50	−9979.22	0.009274
	cg18752880	C1q and tumor necrosis factor related protein 3	C1QTNF3	−1.03E+06	0.028981
	cg24691461	cerebral cavernous malformation 2-like, CCM2L	C20orf160	−4898.6	0.00563
	cg02654291	chromosome 9 open reading frame 64	C9orf64	−254582	0.016801
	cg21489873	MIR600 host gene (non-protein coding)	C9orf45	−2.41E+19	0.049695

B. 33 Genes under-expressed in CAF compared to NF

Function	Probeset	Gene name	Gene Symbol	Fold Change	P value
	cg15679095	GLTSCR1-like	KIAA0240	−6.85E+31	0.034122
	cg02077702	immunoglobulin superfamily containing leucine-rich repeat	ISLR	−1.13E+23	0.030866
	cg19170321	Calpain 3, p94	CAPN3	−4.66E+13	0.0233799