

## Imaging, Diagnosis, Prognosis

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## Hypoxia-Driven Gene Expression Is an Independent Prognostic Factor in Stage II and III Colon Cancer Patients

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## Abstract

**Purpose:** Hypoxia is considered a major microenvironmental factor influencing cancer behavior. Our aim was to develop a hypoxia-based gene score that could identify high and low risk within stage II and III colon cancer patients.

**Experimental Design:** Differential gene expression of CaCo-2 colon cancer cells cultured in chronic hypoxia versus normoxia was tested for correlation with prognostic variables in published microarray datasets. These datasets were further used to downsize and optimize a gene score, which was subsequently determined in paraffin-embedded material of 126 patients with colon cancer treated in our center.

**Results:** In the CaCo-2 cells, 923 genes with a 2-fold change and Limma corrected  $P \leq 0.0001$  were found differentially expressed in hypoxia versus normoxia. We identified 21 genes with prognostic value and overlapping in three different training sets and ( $n = 224$ ). With a fourth published dataset ( $n = 177$ ), the six-gene Colon Cancer Hypoxia Score (CCHS) was developed. Patients with low CCHS showed a significant better disease-free survival at three years (77.3%) compared with high CCHS patients (46.4%; log-rank,  $P = 0.006$ ). This was independently confirmed in an external patient cohort of 90 stage II patients (86.9% vs. 52.2%;  $P = 0.001$ ).

**Conclusions:** Hypoxia-driven gene expression is associated with high recurrence rates in stage II and III colon cancer. A six-gene score was found to be of independent prognostic value in these patients. Our findings require further validation and incorporation in the current knowledge on molecular classification of colon cancer. *Clin Cancer Res*; 20(8); 2159–68. ©2014 AACR.

## Introduction

In Western countries, colorectal cancer is the third most common cancer in both men and women and the second leading cause of cancer-related death, accounting for approximately 500,000 deaths annually (1). Tumor stage (American Joint Committee on Cancer; AJCC) at presentation is the main factor for therapeutic decisions and prognostic estimates. Patients with stage II disease generally undergo surgery only, whereas in stage III, adjuvant chemotherapy after resection of the affected bowel segment and attached lymph nodes is the preferred treatment option (2). However, data from historical trials show that 60% of

stage III patients do not recur following surgery only, whereas 20% of stage II patients do have recurrent disease after resection (3, 4). These findings have led to an extensive search for prognostic markers that could classify patients with colorectal cancer more accurately, reducing over- or undertreatment. The finding that stage II patients with a high degree of tumor microsatellite instability (MSI-H) are at very low risk of recurrence helps guiding treatment (5). Driven by the success of this approach in breast cancer (6), extensive research has led to the development of dozens of prognostic gene signatures for colon cancer (7). Although used by some oncologists, gene signatures are currently neither approved by the U.S. Food and Drug Administration nor adopted by any colorectal cancer treatment guideline (8).

In this study, the aim was to develop a robust prognostic gene score to improve the subclassification of stage II and III colon cancer patients using a mechanism-based approach previously described for hepatocellular carcinoma (9). It is now recognized that microenvironmental factors such as hypoxia play a role in tumor behavior. Exposed to low oxygen supplies, cancer cells become more aggressive, invasive, and resistant to therapy (10).

*In vitro*, we identified the differentially expressed genes under chronic hypoxia versus normoxia. Using bioinformatics on three published sets of expression data with

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

Hypoxia is a well-known microenvironmental factor determining tumor behavior. Despite this knowledge, its influence is never taken into account in clinical practice. We looked at gene expression changes in colon cancer cells exposed to chronic hypoxia. The hypoxia-induced expression pattern correlated with the clinical outcome of more than 200 patients with colon cancer in published microarray datasets, confirming the detrimental role of intratumoral hypoxia on prognosis. We further describe the development of a 6-gene Colon Cancer Hypoxia Score (CCHS), which was found prognostic in two additional patient cohorts. Its value was independent of other important variables such as disease stage, differentiation grade, and vascular invasion. Moreover, the CCHS performed excellent in both fresh-frozen and paraffin-embedded material.

corresponding clinical information, we could identify 21 genes, related to both hypoxia and prognosis. Further optimization of the model led to a Colon Cancer Hypoxia Score (CCHS) of six genes, which we evaluated in formalin-fixed, paraffin-embedded (FFPE) tissue of our own patient cohort as well as in an external dataset.

## Materials and Methods

### Cell culture and RNA isolation

Method details are described in the Supplementary Files. Briefly, as *in vitro* model, we used the human adenocarcinoma cell line CaCo-2 (HTB-37; American Type Culture Collection). Cells were grown in a humidified incubator (Sanyo MCO-18M O<sub>2</sub>/CO<sub>2</sub> incubator; 5% CO<sub>2</sub> at 37°C) in MEM medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mmol/L sodium pyruvate.

For the determination of chronic hypoxia conditions by real-time PCR (RT-PCR), cells were seeded in 25 cm<sup>2</sup> culture flasks (10<sup>6</sup> cells/flask) that were placed in either 2% O<sub>2</sub> or 20% O<sub>2</sub> after 24-hour attachment. All culture conditions were performed in triplicate and cells were collected for RNA isolation at 0, 10, 24, 48, and 72 hours.

For microarray analysis, cells were seeded at 3 × 10<sup>6</sup> in 75 cm<sup>2</sup> tissue culture flasks (*n* = 4) at 20% O<sub>2</sub> and were grown until 70% confluence. After reaching near-confluence, two flasks were placed in a humidified incubator with hypoxic conditions (2% O<sub>2</sub>, 5% CO<sub>2</sub> at 37°C) for 72 hours, whereas two other flasks remained in normoxic conditions (20% O<sub>2</sub>).

Cells were harvested after trypsin treatment and mixed with TRIzol (Invitrogen). RNA was isolated with the RNeasy Kit (Qiagen) according to the manufacturer's instructions.

### RT-PCR

RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen). The PCR reaction was carried out using TaqMan

Universal PCR Master Mixture and Assays-on-Demand Gene Expression products (Applied Biosystems). The primers used are listed in Supplementary Table S1. β-2 microglobulin was used as housekeeping gene. RT-PCR amplification and data analysis were performed using the A7500 Fast Real-Time PCR System (Applied Biosystems). The ΔΔC<sub>t</sub> method was used to determine the relative gene expression levels.

### Microarray

Samples were hybridized on dual color Agilent's Human Whole Genome Oligo Microarray (Cat# G4112F, Agilent) that contained 44 k 60-mer oligonucleotide probes representing around 41,000 well-characterized human transcripts. Sample processing, quality control, and microarray data analysis are described in detail in the Supplementary Materials and Methods. To identify the highly significant differentially expressed genes under chronic hypoxic conditions, probes with a 2-fold change and a corrected *P* value less than 0.0001 after multiple testing corrections were selected (11). Results were confirmed by performing RT-PCR on selected genes.

### Correlation of the hypoxia gene expression pattern with prognosis in external datasets

Biomarker development and evaluation were done following the REMARK guidelines for tumor marker studies (12). To determine the clinical relevance of the hypoxia gene expression pattern, we compared our findings with published microarray datasets containing corresponding clinical information.

We considered four datasets of patients with colon carcinoma, published in Gene Expression Omnibus (GEO; Table 1), all based on the GeneChip Human Genome U133 Plus 2.0 array from Affymetrix. In Jorissen and colleagues, 63 microsatellite stable (MSS) and 11 unstable (MSI) colorectal cancer samples from the Royal Melbourne Hospital (Parkville, Australia) were investigated (13). In Aronow and colleague, recurrence status was available for 100 patients with colon cancer after exclusion of mucosa samples (14). A total of 232 patients with colon cancer and known clinical outcome were included in the study by Smith and colleagues (15). This set was divided into 55 samples from Vanderbilt Medical Center (VMC; Nashville, TN) and 177 samples from the Moffitt Cancer Center (MCC; Tampa, FL).

All datasets were independent of one another, except for the dataset of Aronow and colleagues, which shows partial overlap with MCC samples (own observations). For this reason, we used the datasets of Jorissen and Aronow and the VMC subset of Smith to determine the prognostic value of the hypoxia gene expression pattern with a global test by Goeman and colleagues (16).

Subsequently, overlap between the hypoxia gene expression pattern and the three datasets was used to downsize the number of genes (prognostic signature; see Fig. 1).

The MCC subset of Smith was used to optimize this signature by backward regression analysis using SPSS package 19 (IBM). A probability of *F* with a *P* value of 0.05 was set

**Table 1.** Clinical overview of published microarray datasets used for global performance testing of the hypoxia expression pattern and for development and optimization of the CCHS

| Author Reference       | Jorissen 13        | Aronow 14                                   | Smith 15           |                  |
|------------------------|--------------------|---|--------------------|------------------|
|                        |                    |   | VMC database       | MCC database     |
| Dataset ID             | GSE13294           | GSE5206                                     | GSE17537           | GSE17536         |
| Used for               | Training signature | Training signature                          | Training signature | Developing score |
| Array type             |                    | Affymetrix Human Genome U133 Plus 2.0 Array |                    |                  |
| Sample type            | Fresh frozen       | Fresh frozen                                | Fresh frozen       | Fresh frozen     |
| N samples              | 74                 | 105   | 55                 | 177              |
| N colon cancer         | 74                 | 100   | 55                 | 177              |
| Rectal cancer included | Yes                | Yes   | Yes                | Yes              |
| N other                | —                  | 5 normal mucosa                             | —                  | —                |
| Age (median, y)        | NA                 | 66  | 62                 | 66               |
| Min.                   |                    | 26  | 23                 | 26               |
| Max.                   |                    | 92  | 94                 | 92               |
| Sex (M/F)              | NA                 | —   | —                  | —                |
| Male                   | —                  | 46 (46%)                                    | 26 (47%)           | 96 (54%)         |
| Female                 | —                  | 54 (54%)                                    | 29 (53%)           | 81 (46%)         |
| AJCC stage             | NA                 | —   | —                  | —                |
| I                      | —                  | 15 (15%)                                    | 4 (7%)             | 24 (14%)         |
| II                     | —                  | 29 (29%)                                    | 15 (27%)           | 57 (32%)         |
| III                    | —                  | 33 (33%)                                    | 19 (35%)           | 57 (32%)         |
| IV                     | —                  | 20 (20%)                                    | 17 (31%)           | 39 (22%)         |
| NA                     | —                  | 3 (3%)                                      | 0                  | 0                |
| Differentiation        | NA                 | —   | —                  | —                |
| Good                   | —                  | 8 (8%)                                      | 1 (2%)             | 16 (9%)          |
| Moderate               | —                  | 78 (78%)                                    | 32 (58%)           | 134 (76%)        |
| Poor                   | —                  | 10 (10%)                                    | 3 (5%)             | 27 (15%)         |
| NA                     | —                  | 4 (4%)                                      | 19 (35%)           | 0                |
| Recurrence             | NA                 | —   | —                  | —                |
| Yes                    | —                  | 23 (23%)                                    | 19 (34%)           | 36 (24%)         |
| No                     | —                  | 77 (77%)                                    | 36 (66%)           | 109 (62%)        |
| NA                     | —                  | —   | —                  | 32 (18%)         |
| Median FU (mo.)        | NA                 | NA  | 50.2               | 42.3             |
| Min.                   | —                  | —   | 0.4                | 0.9              |
| Max.                   | —                  | —   | 111                | 142.5            |
| MSI                    | —                  | NA  | NA                 | NA               |
| MSS                    | 63 (85%)           | —   | —                  | —                |
| MSI                    | 11 (15%)           | —   | —                  | —                |

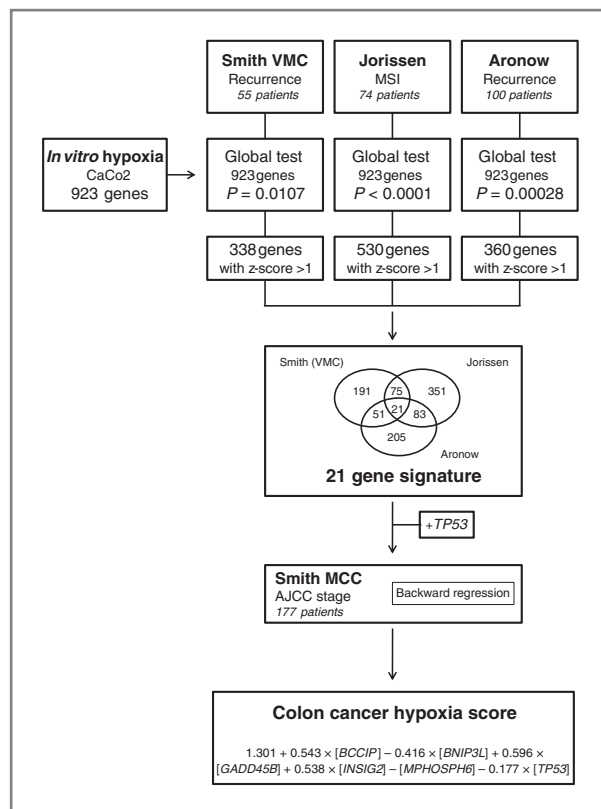
Abbreviation: NA, not available.

as the threshold for entry and removal. This way, we selected those genes that contributed the most to the model and enriched them with a coefficient, which describes the relative contribution of the gene. The obtained score was named the "Colon Cancer Hypoxia Score."

#### Prospective evaluation of the Colon Cancer Hypoxia score

**The Leuven cohort.** A total of 162 patients with stage II or III colon cancer treated in our center between 2004 and 2006 were selected to test the performance of the CCHS. Patients with missing clinical data, patients with rectal

cancer, and those who did not survive at least 1 month after surgery were excluded. For this study, we used two parallel slides of FFPE material per patient. The first slide was hematoxylin and eosin stained, and the tumor tissue marked by a pathologist. Tumor was then dissected from nontumorous tissue on the second slide and subsequently deparaffinized. RNA was extracted using a modified RNeasy FFPE protocol (Supplementary Materials and Methods). Quantification, sizing, and quality control were performed with the Bioanalyzer platform (Agilent). Only samples with at least 50% of RNA fragments longer than 200 bases were selected. Samples were analyzed for expression of selected



**Figure 1.** Process to obtain the CCHS. Overlap between three published datasets and the hypoxia gene expression pattern in CaCo-2 cells was used to downsize the number of genes to 21. After adding *TP53*, a backward regression analysis was performed to obtain a six gene score.

genes by the nCounter technique (NanoString Technologies) which gives a direct read-out of transcripts without the use of enzymatic reactions (17). The used CodeSet is described in Supplementary Table S2.

**The Amsterdam cohort.** The AMC-AJCCII-90 dataset consists of gene expression data on fresh-frozen tumor tissue of 90 stage II patients treated in the Academic Medical Center (AMC) in Amsterdam between 1997 and 2006. The Affymetrix Human Genome U133 Plus 2.0 Array was used as microarray platform (GSE33113; ref. 18).

### Statistical analysis

All data are presented as median with range. A  $P < 0.05$  was considered significant. To determine an optimal cutoff value for our CCHS, a receiver operating characteristic curve was used. For the survival analysis, Kaplan–Meier survival curves and univariate and multivariate Cox proportional HR were used. All statistics were performed using SPSS package 19 (IBM).

## Results

### Dynamics in gene expression in CaCo-2

To investigate the dynamics of hypoxia-related gene expression in our *in vitro* model and to determine the optimal timing for microarray analysis, we performed

RT-PCR at different time points for a set of eight representative genes known to be responsive to hypoxia. Gene expression was tested at 0, 10, 24, 48, and 72 hours in hypoxia (2%  $O_2$ ) versus normoxia. It should be noted that when CaCo-2 cells were kept beyond 72 hours, their cultures tended to become super-confluent with partly detachment of the cells resulting in an uncontrolled fluctuation of the gene expression levels (data not shown). Most of the mRNA expression levels investigated showed a dynamic curve over time at 2%  $O_2$  with exception of *BCL2* that did not change significantly at any of the time points investigated (Supplementary Fig. S1). These observations support the assumption that the acute hypoxic state (up to 24 hours) has a different gene expression pattern compared with the more chronic state (72 hours). As such, we chose the latter condition for our microarray experiment.

### Microarray: primary data analysis and development of a Colon Cancer Hypoxia Score

Using Agilent technology, in CaCo-2 cells cultured for 72 hours at either 20% oxygen or in hypoxic conditions at 2% oxygen, a total of 37,707 spots showed a representative signal of which 3,389 with a  $\log_2$  fold-change  $>1$  or  $<-1$  and a Limma corrected  $p$ -value  $<0.05$ . These microarray data are available at NCBI under number GSE31079.

For the development of our gene signature, we started with the top 923 differentially expressed genes (with a  $\log_2$  fold-change  $>1$  or  $<-1$  and a Limma corrected  $P < 0.0001$ ): the hypoxia gene expression pattern. Of those 923 genes, 704 were upregulated and 219 downregulated in hypoxic conditions. The top altered genes and significant KEGG pathways involved are listed in Supplementary Tables S3 and S4.

Subsequently, the Goeman global test showed that the overall expression pattern of these hypoxia genes was significantly related to the prognostic factor considered for each of the three training datasets ( $P < 0.0001$  for Jorissen;  $P = 0.00028$  for Aronow; and  $P = 0.0107$  for Smith VMC).

Next, when only keeping the significant genes with a  $z$ -score, above 1,530 genes remained for the dataset of Jorissen, 360 genes for Aronow, and 338 genes for Smith VMC. Finally, genes for which the direction of altered expression did not correspond to the direction observed *in vivo* in at least two out of three datasets were removed. With this approach, we were able to downsize the number of genes to twenty-one, found to overlap between the three training datasets (Fig. 1). In this gene set of 21 genes, 17 genes were upregulated in hypoxic conditions and four were downregulated (Table 2).

CaCo-2 cells contain a *TP53* mutation resulting in undetectable p53 protein levels (19). As such, *TP53* was not considered early in the development of our gene signature despite being clinically of potential prognostic importance in colon cancer (20). For these reasons, *TP53* was added afterward to the *in vitro*-derived 21 hypoxia cancer genes.

Using a backward linear regression analysis with the 21 genes and *TP53* as independent variables and the AJCC disease stage of patients in a fourth dataset (Smith MCC) as



**Table 2.** Overview of the 21 gene signature with their relative direction *in vitro* under hypoxia and cellular processes involved

| Official gene symbol | Direction <i>in vitro</i> | Gene name  | Processes involved      | Used for CCHS |
|----------------------|---------------------------|--|-------------------------|---------------|
| BCCIP                | ▼                         | BRCA2 and CDKN1A-interacting protein isoform C                       | DNA metabolic/repair    | ✓             |
| BNIP3L               | ▲                         | BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like        | Apoptosis               | ✓             |
| BTBD14A              | ▲                         | Nucleus accumbens-associated protein 2                               | Unknown                 | —             |
| DDIT4                | ▲                         | DNA damage-inducible transcript 4 protein                            | Apoptosis/hypoxia       | —             |
| ERRFI1               | ▲                         | ERBB receptor feedback inhibitor 1                                   | GTPase regulation       | —             |
| FAM46C               | ▼                         | Hypothetical protein LOC54855  | Unknown                 | —             |
| GADD45B              | ▲                         | Growth arrest and DNA damage-inducible protein GADD45 beta           | Apoptosis               | ✓             |
| HBP1                 | ▲                         | HMG box-containing protein 1   | Cell cycle              | —             |
| ING5                 | ▼                         | inhibitor of growth protein 5  | DNA metabolic/repair    | —             |
| INSIG2               | ▲                         | insulin-induced gene 2 protein                                       | ER-nuclear signaling    | ✓             |
| KLF7                 | ▲                         | Krüppel-like factor 7  | Cell morphogenesis      | —             |
| MPHOSPH6             | ▼                         | M-phase phosphoprotein 6   | Cell cycle              | ✓             |
| NNMT                 | ▲                         | Nicotinamide N-methyltransferase                                     | N-methyltransferase     | —             |
| P4HA1                | ▲                         | Prolyl 4-hydroxylase subunit $\alpha$ -1 isoform 1 precursor         | Metabolic processes     | —             |
| P4HA2                | ▲                         | Prolyl 4-hydroxylase subunit $\alpha$ -2 isoform 1 precursor         | Metabolic processes     | —             |
| PLAUR                | ▲                         | Urokinase plasminogen activator surface receptor isoform 3 precursor | Metabolic processes     | —             |
| RHO                  | ▲                         | rho-related GTP-binding protein RhoU                                 | Cell cycle              | —             |
| TMCC1                | ▲                         | Transmembrane and coiled-coil domains protein 1 isoform a            | Transmembrane signaling | —             |
| UBE2H                | ▲                         | Ubiquitin-conjugating enzyme E2 H isoform 1                          | Proteolysis             | —             |
| ULBP2                | ▲                         | NKG2D ligand 2 precursor   | Transmembrane signaling | —             |
| XIAP/BIRC4           | ▲                         | Baculoviral IAP repeat-containing protein 4                          | Apoptosis               | —             |
| TP53                 | NA                        | Tumor protein p53  | Apoptosis/cell cycle    | ✓             |

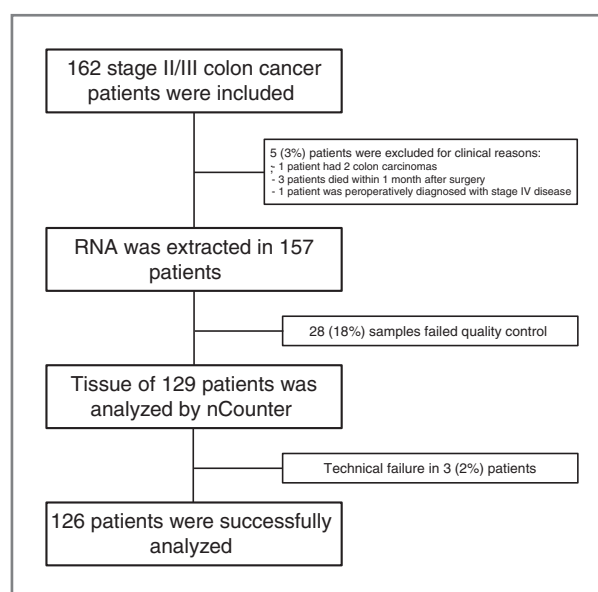
NOTE: *TP53* was added to the signature and eventually incorporated in the CCHS.

dependent variable, six genes showed the highest contribution to the model. These genes with their corresponding coefficient form the CCHS (Fig. 1). The score consists of the expression value of *BCCIP*, *GADD45B*, *INSIG2*, *BNIP3L*, *MPHOSPH6*, and *TP53*. The latter three have an inverse effect on the score value. All genes are involved in processes related to cell cycle, apoptosis, and DNA repair.

### Prospective evaluation of CCHS

Of the 162 stage II/III patients treated in our center between 2004 and 2006, 129 were selected for nCounter analysis after exclusion of clinical aberrant cases and quality control (Fig. 2). Gene expression level of the CCHS genes was successfully obtained in 126 samples (97.7%). The known clinical and pathologic features of these patients are summarized in Table 3. Estimated median disease-free survival (DFS) times were 66.2 months [95% confidence interval (CI), 60–71.5] and 46 months (95% CI, 37.5–54.5) for stage II and stage III patients, respectively.

Using an optimal cutoff (see Supplementary Fig. S2), patients with a low CCHS showed to have significant better DFS at 3 years compared with those with a high CCHS (77.3% vs. 46.4%, respectively;  $P = 0.006$ ). These DFS rates remained unchanged at 5 years. Median DFS was 62.2



**Figure 2.** Inclusion and selection process of the stage II/III colon cancer patients in our center. Of the 162 patients with colon cancer included in this study, after clinical and technical selection, the expression levels of 126 patients were successfully determined.

**Table 3.** Clinical and pathologic features of the Leuven cohort as well as the AMC-AJCCII-90 dataset (16)

|                            | Leuven cohort |            |             | AMC-AJCC-II |
|----------------------------|---------------|------------|-------------|-------------|
|                            | All patients  | Stage II   | Stage III   |             |
| Patient number             | 126           | 71         | 55          | 90          |
| Age (median, y)            | 71.2          | 70         | 72.9        | 73.4        |
| Min. (y)                   | 35.9          | 35.9       | 38.8        | 34.6        |
| Max. (y)                   | 92.9          | 92.9       | 86.2        | 95.1        |
| Sex                        |               |            |             |             |
| Male                       | 67 (53%)      | 34 (48%)   | 33 (60%)    | 42 (47%)    |
| Female                     | 59 (47%)      | 37 (52%)   | 22 (40%)    | 48 (53%)    |
| Localization               |               |            |             |             |
| Right-sided tumor          | 63 (50%)      | 39 (55%)   | 24 (43.5%)  | 48 (53%)    |
| Left-sided tumor           | 63 (50%)      | 32 (45%)   | 31 (56.5%)  | 42 (47%)    |
| T-stage                    |               |            |             | NA          |
| T2                         | 4 (3%)        | 0 (0%)     | 4 (7%)      | —           |
| T3                         | 109 (86.5%)   | 67 (94%)   | 42 (76.5%)  | —           |
| T4                         | 13 (10.5%)    | 4 (6%)     | 9 (16.4%)   | —           |
| N-stage                    |               |            |             | NA          |
| N0                         | 71 (56%)      | 71 (100%)  | 0 (0%)      | —           |
| N1                         | 39 (31%)      | 0 (0%)     | 39 (71%)    | —           |
| N2                         | 16 (13%)      | 0 (0%)     | 16 (29%)    | —           |
| AJCC stage                 |               |            |             |             |
| II                         | 71 (56%)      | 71 (100%)  | 0 (0%)      | 90 (100%)   |
| III                        | 55 (44%)      | 0 (0%)     | 55 (100%)   | 0 (0%)      |
| Mucinous type              |               |            |             | NA          |
| Yes                        | 36 (29%)      | 22 (31%)   | 41 (74, 5%) | —           |
| No                         | 90 (71%)      | 49 (69%)   | 14 (25, 5%) | —           |
| Differentiation            |               |            |             | NA          |
| Good                       | 14 (11%)      | 7 (10%)    | 7 (13%)     | —           |
| Moderate                   | 63 (50%)      | 40 (56.5%) | 23 (42%)    | —           |
| Poor                       | 45 (36%)      | 20 (28%)   | 25 (45%)    | —           |
| Undifferentiated           | 1 (1%)        | 1 (1.5%)   | 0 (0%)      | —           |
| Not available              | 3 (2%)        | 3 (4%)     | 0 (0%)      | —           |
| Peritoneal invasion        |               |            |             | NA          |
| Yes                        | 1 (1%)        | 1 (1%)     | 0 (0%)      | —           |
| No                         | 125 (99%)     | 70 (99%)   | 55 (100%)   | —           |
| Extramural venous invasion |               |            |             | NA          |
| Yes                        | 36 (28.6%)    | 11 (15%)   | 25 (45%)    | —           |
| No                         | 81 (64.3%)    | 59 (84%)   | 22 (40%)    | —           |
| NA                         | 9 (7.1%)      | 1 (1%)     | 8 (15%)     | —           |
| Resection margin           |               |            |             | NA          |
| Positive                   | 1 (1%)        | 0 (0%)     | 1 (2%)      | —           |
| Negative                   | 124 (98%)     | 71 (100%)  | 53 (96%)    | —           |
| NA                         | 1 (1%)        | 0 (0%)     | 1 (2%)      | —           |
| Bowel perforation          |               |            |             | NA          |
| Yes                        | 5 (4%)        | 3 (4%)     | 2 (4%)      | —           |
| No                         | 121 (96%)     | 68 (96%)   | 53 (96%)    | —           |
| Examined lymph nodes       |               |            |             | NA          |
| 12 or more                 | 83 (66%)      | 49 (69%)   | 34 (62%)    | —           |
| Less than 12               | 42 (33%)      | 22 (31%)   | 20 (36%)    | —           |
| NA                         | 1 (1%)        | 0 (0%)     | 1 (2%)      | —           |
| Adjuvant chemotherapy      |               |            |             | NA          |
| Yes                        | 63 (50%)      | 50 (70.4%) | 42 (76.5%)  | —           |
| No                         | 63 (50%)      | 21 (29.6%) | 13 (23.5%)  | —           |

*(Continued on the following page)*

**Table 3.** Clinical and pathologic features of the Leuven cohort as well as the AMC-AJCCII-90 dataset (16) (Cont'd)

|                | Leuven cohort |          |           | AMC-AJCC-II |
|----------------|---------------|----------|-----------|-------------|
|                | All patients  | Stage II | Stage III |             |
| Relapse        |               |          |           | —           |
| Yes            | 35 (28%)      | 12 (17%) | 23 (42%)  | 19 (21%)    |
| No             | 91 (72%)      | 59 (83%) | 32 (58%)  | 71 (79%)    |
| Median FU (mo) | 45.42         | 49.14    | 23.1      | 39.3        |
| Min. (mo)      | 1.3           | 1.3      | 1.5       | 1.6         |
| Max. (mo)      | 76.4          | 76.4     | 73.9      | 120         |

Abbreviations: Median FU, median follow-up defined as time to relapse or last contact; NA, not available.

months (95% CI, 56.6-67.7) and 42.7 months (95% CI, 33.4-52) for CCHS low and CCHS high patients, respectively ( $P = 0.006$ ; Fig. 3A). Patients with a favorable CCHS were more likely to be alive at 3 years (estimated overall survival 81.5% vs. 66.6%;  $P = 0.044$ ) and at 5 years (71.9% vs. 59.1%;  $P = 0.036$ ).

When disease AJCC stage was taken into account, survival analysis could identify three groups with significant difference in survival (Fig. 3B). Patients with stage II disease and unfavorable CCHS had almost similar disease behavior as stage III patients with a good (low) CCHS. Moreover, the CCHS could identify a subgroup of stage III patients with an estimated recurrence rate at 3 years of 75% and a median DFS of only 28.2 months (95% CI, 16-40.4).

The characteristics of the 90 patients of the Amsterdam cohort are listed in Table 3. Estimated DFS time for the whole cohort was 95.5 months (95% CI, 85.8-105.2). Similar to the Leuven patient cohort, the CCHS identified two subgroups of stage II patients with different DFS at 3 years (86.9% vs. 52.2%;  $P < 0.001$ ) and 5 years (86.9% vs. 52.2%;  $P < 0.001$ ). Median DFS was 102.5 months (95% CI, 93.1-111.9) versus 55.4 months (95% CI, 38.7-72;  $P = 0.001$ ; Fig. 3C). HR for recurrence in CCHS high versus CCHS low patients was 3.970 (95% CI, 1.6-9.8;  $P = 0.004$ ).

Subsequently, a univariate Cox regression analysis was performed on the Leuven cohort analyzing all known clinical and pathologic variables. Four variables with significant prognostic value were included in a multivariate analysis. The multivariate Cox regression model confirmed that the CCHS is a predictor for recurrence independent of disease stage, venous invasion, and the number of examined lymph nodes (Table 4).

## Discussion

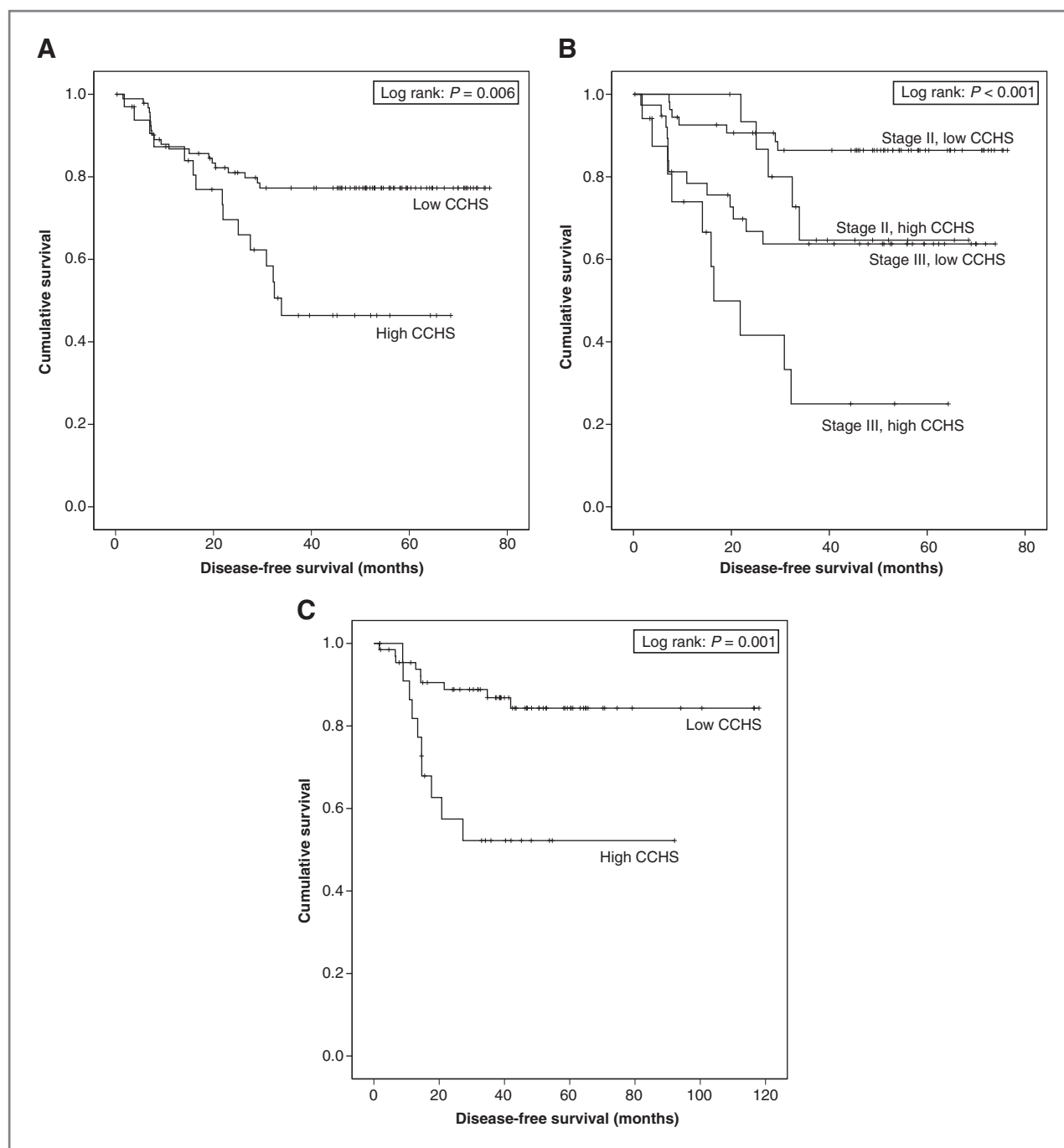
Colon cancer is associated with high morbidity and mortality, and prognostic subclassification of stage II and III patients remains an ongoing clinical challenge. We describe the development and testing of a mechanism-based prognostic gene score. Hypoxia as microenvironmental factor is well known for its influence on cancer behavior. If hypoxia does not induce cell death, it increases tumor

aggressiveness, invasiveness, and metastatic potential mainly through activation of the hypoxia-inducible factor (HIF) pathway (10). Despite this knowledge, hypoxia is seldom taken into account in the clinical setting, in part, due to the lack of good endogenous hypoxia markers (21). HIF overexpression has been proven associated with prognosis, but HIF itself is not considered to be a hypoxia marker exclusively due to the fact that it can be activated in various settings, including oncogene drive (22). Our method shows a correlation between the altered expressions of hundreds of genes under hypoxic conditions *in vitro*, with that of tumors in published microarray datasets with clinical data available. This confirms the role of intratumoral hypoxia in disease behavior as was previously shown in HCC (9).

Colon cancer is subject to extensive global scientific research and many gene signatures and scores have been developed in the past mostly based on hierarchical clustering. Some of these signatures have made it to commercially available tests. Although a promising approach to capture the complexity of cancer biology, few signatures show good power in external patient cohorts (7). We tried to address these limitations by using a mechanism-driven approach and involving different datasets in the development of our signature. This resulted in a workable amount of genes derived from data of more than 400 patient samples. Moreover, we hypothesized that the optimization process with a backward regression model has added power to the prognostic value of the genes.

p53 is a known important tumor suppressor in colon cancer and its pathway is modulated by hypoxia (23). This is further supported by our findings that, *in vitro* under hypoxic conditions, the p53 pathway is a highly significant altered KEGG pathway (Supplementary Table S4 and Supplementary Fig. S3). For this reason and those stated above, *TP53* was appended to the gene list in the knowledge that the backward regression analysis would eliminate this gene immediately in the absence of an added value to the model. The fact that it was withheld in the CCHS suggests that *TP53* gene expression is of prognostic value for patients with colon cancer.

Evaluation of the CCHS was done in FFPE material. For mRNA level measurements, this type of material often



**Figure 3.** A, Kaplan-Meier curve for DFS in the Leuven cohort ( $n = 126$ ). B, AJCC disease stage and CCHS divide patients in three groups with significant differences in DFS. When the patients of AJCC with stage II disease and a high CCHS have comparable outcome to patients with stage III disease and a low CCHS. C, Kaplan-Meier curve for DFS in the Amsterdam cohort ( $n = 90$ ).

presents a challenge as it is subjected to RNA degradation. After strict quality control (Fig. 2), the nCounter platform showed excellent performance and reliability for determining the expression levels of all six CCHS genes. Independent of other clinical and pathologic parameters, the CCHS was found to predict relapse in stage II and III colon cancer. In stage II patients, it identified a high-risk subgroup that

might benefit from adjuvant therapy. Furthermore, stage III patients with unfavorable CCHS suffer from high recurrence rates.

Recently, progress has been made in the field of molecular classification of colon cancer based on gene expression clustering, epigenetic markers, and therapy response. This approach led to the proposition of three to six colorectal



**Table 4.** Univariate and multivariate Cox regression analysis on all clinical and pathologic variables in the Leuven cohort

|  | Univariate Cox regression analysis |                  |      |        |       | Multivariate Cox regression analysis |              |      |        |       |
|--|------------------------------------|------------------|------|--------|-------|--------------------------------------|--------------|------|--------|-------|
|  | $\beta$<br>coefficient             | <i>P</i>         | HR   | 95% CI |       | $\beta$<br>coefficient               | <i>P</i>     | HR   | 95% CI |       |
|  |                                    |                  |      | Lower  | Upper |                                      |              |      | Lower  | Upper |
| Age  | 0.03                               | 0.070            | 1.03 | 1.00   | 1.07  |                                      |              |      |        |       |
| Location (left vs. right)                          | 0.13                               | 0.701            | 1.14 | 0.59   | 2.21  |                                      |              |      |        |       |
| Stage (III vs. II)                                 | 1.23                               | <b>0.001</b>     | 3.41 | 1.69   | 6.86  | 0.95                                 | <b>0.017</b> | 2.58 | 1.19   | 5.61  |
| Peritoneal invasion (yes vs. no)                   | 1.30                               | 0.203            | 3.66 | 0.50   | 26.89 |                                      |              |      |        |       |
| Extramural venous invasion (yes vs. no)            | 1.60                               | <b>&lt;0.001</b> | 4.95 | 2.43   | 10.07 | 1.17                                 | <b>0.003</b> | 3.21 | 1.50   | 6.89  |
| Resection margin (positive vs. negative)           | 1.47                               | 0.149            | 4.36 | 0.59   | 32.24 |                                      |              |      |        |       |
| Bowel perforation (yes vs. no)                     | 0.70                               | 0.336            | 2.02 | 0.48   | 8.45  |                                      |              |      |        |       |
| Mucinous type (yes vs. no)                         | −0.27                              | 0.506            | 0.76 | 0.35   | 1.68  |                                      |              |      |        |       |
| Differentiation                                    |                                    | 0.856            |      |        |       |                                      |              |      |        |       |
| Differentiation (moderate vs. good)                | 0.38                               | 0.541            | 1.46 | 0.43   | 4.97  |                                      |              |      |        |       |
| Differentiation (poor vs. good)                    | 0.66                               | 0.296            | 1.94 | 0.56   | 6.77  |                                      |              |      |        |       |
| Adjuvant chemotherapy (yes vs. no)                 | 0.19                               | 0.587            | 1.20 | 0.62   | 2.35  |                                      |              |      |        |       |
| CCHS (high vs. low)                                | 0.91                               | <b>0.008</b>     | 2.49 | 1.27   | 4.86  | 0.95                                 | <b>0.013</b> | 2.58 | 1.23   | 5.43  |
| Lymph nodes examined (12 or more vs. less than 12) | −0.73                              | <b>0.034</b>     | 0.48 | 0.25   | 0.95  | −0.40                                | 0.283        | 0.67 | 0.33   | 1.39  |

NOTE: CCHS is an independent predictor of disease recurrence.

cancer subtypes (18, 24, 25). De Sousa and colleagues identified two classes which seem to represent the known subgroups of MSI/CIMP and chromosomal-unstable tumors, respectively (18). A third class, previously unidentified and related to serrated polyps, was associated with a poor prognosis. Tumors of this subgroup have stemness features, which is in line with others who have isolated a stem cell or epithelial-to-mesenchymal transition associated subgroup of patients with colon cancer with high recurrence rates (24–26). We see a striking overlap between the KEGG pathways involved in adapting to hypoxic conditions and those pathways attributed to the stem cell-like phenotype proposed by Marisa and colleagues (ref. 25; Supplementary Table S4). Further prospective validation in a larger patient cohort is needed to clarify whether a high CCHS is indicative for the stemness subtype. If so, the limited number of genes together with the performance in FFPE samples renders our CCHS a potential clinical tool.

This study has some limitations. Unfortunately, at the time of study onset, extensive clinical data were not available for some of the published datasets used for CCHS development. This forced us to use surrogate parameters such as MSI which could influence the power of the obtained score. During study design, after careful evaluation of the available information, considerable overlap between different datasets published in the GEO was noticed. For example, most samples of the dataset provided by Aronow and colleagues (GSE5206) have also been included in both GSE14333 and GSE17536 (own observations). This requires great caution when designing studies with this widely used published data. In our case, we

compared the individual patient data of the datasets and found that there was a partial overlap between the database by Aronow and Smith MCC. In our study design, the latter was only involved in optimization of the gene signature and this overlap should therefore not affect our conclusions.

When testing the performance of the CCHS, we excluded rectal cancer. Despite the obvious similarities between colon and rectal cancer, we wanted to exclude the differences in treatment modalities as a confounder. As such, our results cannot be extrapolated to these patients.

Finally, our study was designed to evaluate the CCHS as a prognostic marker. Further research is needed to determine whether the score has predictive abilities, that is, potency to identify patients likely to benefit from adjuvant treatment.

In conclusion, we discovered that the gene expression pattern of CaCo-2 cells cultured in hypoxia shows good correlation with clinical parameters of patients with colon cancer in published microarray datasets. This confirms the importance of intratumoral hypoxia in disease behavior. From this point, a six-gene CCHS was developed which proved to be an independent prognostic biomarker for relapse in stage II and III colon cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Clinical Cancer Research

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