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Research Article

Five-Gene Expression Signature Associated With Acquired FOLFIRI Resistance and Survival in Metastatic Colorectal Cancer

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ABSTRACT

FOLFIRI, a combination of folinic acid, 5-fluorouracil, and irinotecan, is one of the recommended first-line chemotherapeutic treatments for metastatic colorectal cancer. Unfortunately, acquired FOLFIRI resistance represents a common obstacle in the treatment of metastatic colorectal cancer patients. Thus, we aimed to identify mechanisms, gene alterations, and gene expression signatures contributing to acquired FOLFIRI resistance by mimicking this problem in a cell culture model and subsequent translation in clinical data sets. Three FOLFIRI-resistant colorectal cancer (CRC) cell lines were established by continuous FOLFIRI treatment. Comparative mutation screening (161 genes) and transcriptomics (pathway and differential expression analyses) were performed in parental and resistant cells. Data reconciliation was performed in GSE62322, a clinical FOLFIRI responder data set (intrinsic resistance). Relapse-free survival (RFS) associations of identified differentially expressed genes and potential gene signatures were investigated in 8 clinical CRC data sets. No mutual genetic alterations were found in FOLFIRI-resistant derivatives. Resistant cell lines displayed activation of mitogen-activated protein kinase, immune response, and epithelial-mesenchymal transition pathways. Twelve differentially expressed genes, significantly differentially expressed in at least 2 of the 3 resistant cell lines, were identified. Comparison with GSE62322 and subsequent survival analyses revealed a 5-gene FOLFIRI signature comprised of *CAV2*, *TNC*, *TACSTD2*, *SERPINE2*, and *PERP* that was associated with RFS in multiple data sets including the cancer genome atlas CRC (hazard ratio [HR] =2.634, $P = 4.53 \times 10^{-6}$), in pooled samples of all data sets (all stages [N = 1981]: HR = 1.852, $P = 6.44 \times 10^{-13}$; stage IV [N = 260]: HR = 2.462, $P = 5.22 \times 10^{-9}$). A multivariate Cox regression analysis identified the 5-gene signature as an independent prognostic factor in the cancer genome atlas data set (HR = 1.89, $P = .0202$). Our analyses revealed a 5-gene FOLFIRI resistance signature associated with RFS that may help predict FOLFIRI resistance and thus avoid unnecessary ineffective treatment. Signature members might also represent targets to fight FOLFIRI resistance.

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Introduction

Despite advances in local and systemic treatment as well as extended molecular testing with the potential of targeted therapies, 5-year survival rates in metastatic colorectal cancer (mCRC) only range around 14%.[1] In fact, about 20% of patients initially present with metastases, commonly in the liver, up to 60% of patients develop distant metastases within 5 years, and 75% of patients show recurrence within 18 months after resection of colorectal liver metastases.[2,3] This can be majorly attributed to therapy resistance, which represents a major obstacle to curative treatment of mCRC.[4,5] International guidelines recommend a combination of folinic acid, 5-fluorouracil (5-FU/F) with oxaliplatin (eg, FOLFOX), or irinotecan (eg, FOLFIRI) or both (eg, FOLFOXIRI) as first-line treatment in mCRC with comparable outcomes.[5] However, despite the improvements in survival rates following these chemotherapeutic regimens, intrinsic and acquired chemoresistance represents a major hindrance toward remission.[4,5]

As opposed to intrinsic resistance that stochastically occurs in untreated colorectal cancer (CRC), acquired resistance encompasses genomic (eg, adaptive mutability[6]), transcriptomic,[7,8] as well as epigenetic alterations that cells undergo to evade therapeutic pressure.[3,7-9] Ongoing chemotherapeutic stress not only fosters an increase in genomic instability but can eventually lead to de novo mutations.[6,10] Nevertheless, recent studies also imply an important role of expression changes rather than genomic alterations induced by first-line treatment of mCRC.[7]

A few studies recently identified gene signatures on a transcriptomic level that predict prognosis[11,12] as well as response to monotherapy[13] or the full chemotherapeutic regimen[13-15] in CRC. To the best of our knowledge, only a single clinical study addressed the identification of a gene signature associated with response to FOLFIRI in mCRC based on data generated from therapy-naive primary tumors reflecting primary chemotherapeutic resistance.[14] Preclinical studies utilizing cell culture approaches mimicking acquired resistance to mCRC chemotherapy regimens are rare. In a recent study, comparative transcriptomic analyses in FOLFOXIRI-resistant CRC cell lines identified 3 candidate genes that were not further investigated regarding survival associations in clinical data sets.[16]

To our knowledge, studies investigating the underlying molecular mechanisms of acquired chemotherapeutic resistance are rare, and an mRNA gene signature that may predict therapeutic FOLFIRI resistance as opposed to therapeutic response has yet to be identified. In this respect, a cell culture model mimicking acquired resistance to FOLIFIRI was established, and comparative mutation screening (161 genes) as well as transcriptomics were performed to, first, elucidate molecular mechanisms of acquired chemotherapeutic resistance; second, identify a gene expression signature associated with FOLFIRI resistance; and third, validate our findings in available clinical data sets. Eventually, identification of new biomarkers that predict acquired/secondary therapeutic resistance as well as a better understanding of the underlying mechanisms can help not only avoid unnecessary side effects of ineffective treatment but may also allow to target resistance mechanisms and optimize as well as personalize treatment in a precision medicine approach.

Materials and Methods

Cell Lines and Transient Transfections

All cell lines were obtained from CLS (Cell Lines Service) and DSMZ (German Collection of Microorganisms and Cell Cultures) or through exchange. An in-house short-tandem-repeat profile analysis was used to confirm the identity of cell lines. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin and regularly tested for mycoplasma contaminations using the Mycoplasma Test Kit from Applichem.

Generation of FOLFIRI-Resistant Cell Lines

To establish FOLFIRI-resistant (R) cell lines, FOLFIRI-sensitive parental (P) CRC cells (Colo205, SW480, and HT29) were continuously treated with increasing FOLFIRI concentrations for ~12 months. FOLFIRI components were obtained from the pharmacy of the University Hospital of the Ludwig-Maximilians-University Munich and mixed in the following ratios according to Pozzi et al[17]: 1 (folinic acid): 1 (5-FU (F)): 3 (irinotecan). Initially, the half-maximal inhibitory concentrations (IC_{50}) of the parental cells were determined by cell viability assay. FOLFIRI treatment was started using concentrations of about twice the initial IC_{50} of P-cells. Subsequently, the FOLFIRI IC_{50} was measured every 4 to 6 weeks starting at month 4 for about 12 months as depicted in Figure 1A. FOLFIRI treatment concentrations were adapted according to the determined IC_{50} increase. P-cell derivatives were considered resistant after reaching a resistance index (RI; IC_{50} resistant/IC_{50} parental) of at least 2 (Supplementary Table S1).

Cell Viability Assay—FOLFIRI Sensitivity

FOLFIRI sensitivity (IC_{50}) was determined by treatment of the cells with increasing FOLFIRI concentrations followed by cell viability assay. Parental or resistant cells were detached and seeded in quadruplicates at 2×10^3 cells per well in 96-well plates for subsequent treatment. FOLFIRI or vehicle control was added 24 hours later. Cells were treated for 72 hours before determining cell viability with the AlamarBlue assay. Briefly, 10 μL AlamarBlue reagent (Thermo Fisher Scientific) was added to each well. Fluorescence was measured after 4 hours with a Varioskan plate reader (Thermo Fisher Scientific). GraphPad Prism (v.8.2.1, GraphPad Software, Inc) was used to calculate cell viability and half-maximal inhibitory concentrations (IC_{50}). Statistical differences between dose-response curves were estimated by a 1-way analysis of variance and Bonferroni's multiple comparison post-test approach (GraphPad Prism).

Nucleic Acid Extraction

RNA was extracted from cell lines as described previously using the RNeasy mini kit (Qiagen).[18,19] Genomic DNA (gDNA) was isolated with the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Nucleic acid concentrations were quantified with a Qubit 2.0 fluorometer (Invitrogen) and the Qubit RNA high sensitivity or the Qubit dsDNA high sensitivity Assay kits (both Thermofisher).

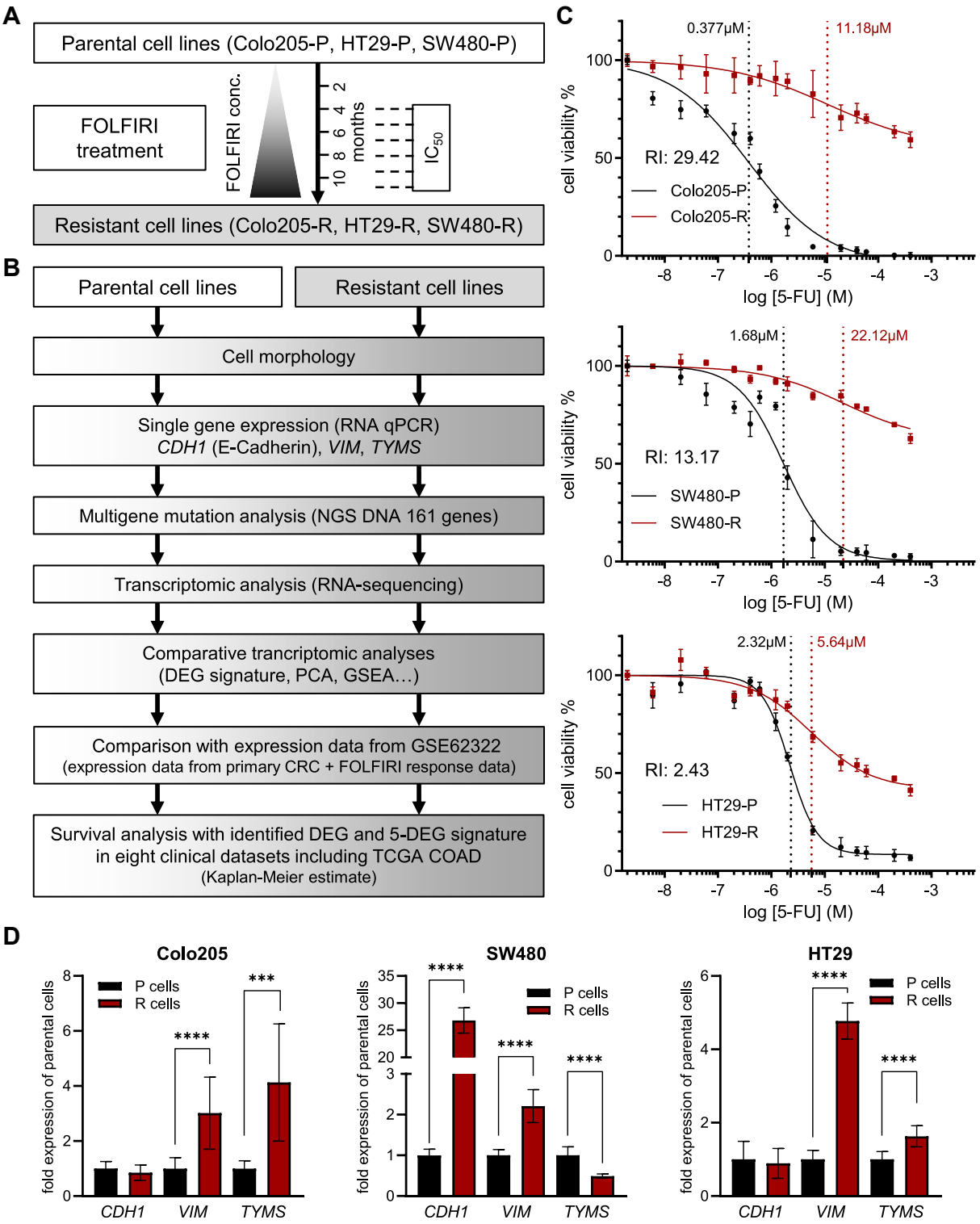

Figure 1.

(A, B) Study design. (A) Establishment of FOLFIRI-resistant (R) CRC cell lines. Indicated parental (P) cells were continuously treated with increasing FOLFIRI concentrations for ~12 months. FOLFIRI IC_{50} was measured by cell viability assay every 4 to 6 weeks starting at month 4. FOLFIRI treatment concentrations were adapted according to the determined IC_{50} increase. (B) Flow chart of the study design to identify a FOLFIRI resistance gene expression signature associated with survival. (C, D) Comparison of parental (P) and established corresponding resistant (R) CRC cell lines Colo205, SW480, and HT29. (C) Cell viability was measured at 72 hours of FOLFIRI treatment at indicated 5-FU concentrations in quadruplicates (error bars indicate SD). IC_{50} (dashed lines) and resistance index (RI) are shown. (D) mRNA expression of *CDH1* (E-Cadherin), *VIM*, *TYMS* in parental and resistant cells was measured by real-time PCR (qPCR). Mean of fold expression (relative to parental cells) from 5 independent cell seedings each determined in triplicates are presented (error bars indicate SD). *P* values were calculated with unpaired *t*-test. ***, $P < .001$; ****, $P < .0001$. COAD, colorectal adenocarcinoma; CRC, colorectal cancer; DEG, differentially expressed gene; GSEA, gene set enrichment analysis; NGS, next generation sequencing; PCA, principal component analysis; TCGA, the cancer genome atlas.

DNA Panel Sequencing and Variant Interpretation

gDNA from P and R cell lines were analyzed with the AmpliSeq for Illumina Oncomine Comprehensive Assay v3 (Illumina), targeting 161 cancer-associated genes (Supplementary Table S2A). Library preparation, subsequent sequencing, and variant calling were performed as described in detail previously.[20] Briefly, an Illumina NextSeq 500 system (NextSeq 500/550 High Output Kits v2.5, Illumina) was used for sequencing according to the manufacturer's protocols. Local Run Manager (Illumina) was used for analysis of the sequencing data followed by annotation of variant call format-files using wANNOVAR[21] and subsequent filtering for relevant mutations with an in-house python script. Sequencing quality metrics are shown in Supplementary Table S2B. Alterations were confirmed with the Integrative Genomics Viewer (IGV, Broad Institute). Variants were judged as relevant based on the interpretation criteria utilized in ClinVar.[22] Additional variant interpretation was performed with pathogenicity prediction algorithms and other publicly available databases (COSMIC,[23] VarSome,[24] dbSNP [https://www.ncbi.nlm.nih.gov/snp/]). Only likely pathogenic and pathogenic mutations as well as variant of unknown significance or not evaluated in ClinVar with a prediction trend of being likely pathogenic) were reported (Supplementary Table S2C). Single-nucleotide variants, multinucleotide variants, small insertions, deletions, indels, and copy number variations were analyzed.

Real-time reverse-transcription PCR Expression Analysis

Total RNA (1 μg) was transcribed into cDNA using Random Hexamer Primer and the RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific). E-Cadherin (*CDH1*), Vimentin (*VIM*), Thymidylate synthase (*TYMS*), and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expressions were analyzed by qPCR using primers, and universal probe library probes (Roche) shown in Supplementary Table S3 and the LightCycler 480 Probes Master mix (Roche). qPCR mixes were analyzed on a Bio-Rad CFX Connect Real-Time PCR Detection System with Bio-Rad CFX Manager Software 3.1 (Bio-Rad Laboratories). Similar PCR efficiencies (>95%) were determined for all investigated genes. Expression quantification was performed with the delta Ct method using *GAPDH* for normalization.

RNA Sequencing, Data Processing, and Analyses

Library preparation for poly(A) RNA sequencing was performed with the NexteraXT kit (Illumina), as described previously.[19] Libraries were sequenced on an Illumina HiSeq 1500 (Illumina) at the Gene Center Munich (Ludwig-Maximilians-University). Sequencing raw data were processed as follows. Demultiplexing and conversion: bcl2fastq2. Subsequent steps were performed with modules available on the Galaxy platform.[25] Quality control: FastQC/MultiQC. FastQ to BAM (Alignment): RNAStar. Counting/Annotation: HTSeq-count. Normalization of expression data, quantification of expression changes, and identification of differentially expressed genes (DEGs) were conducted with Limma (linear models for microarray) and edgeR packages. Benjamini and Hochberg correction was used for the calculation of adjusted *P* values/false discovery rates (FDR).[26] Significantly DEGs were defined by a $\log_2$-fold expression change ($\log_2$FC) ≥ 1 and $P\text{-}_{\text{adjusted}}$ ($P\text{-}_{\text{adj}}$) < .05.

Data Sources and Statistical Analyses

FOLFIRI responder data set (GSE62322[14]) and expression and clinical data of other CRC patient data sets (GSE38832,[27] GSE39084,[28] GSE17538,[29] GSE33113,[30] GSE14333,[31] GSE41258,[32] and GSE39582[33]) were retrieved from Gene Expression Omnibus.[34] The cancer genome atlas (TCGA) colon adenocarcinoma (COAD) and rectal adenocarcinoma expression and clinical data were obtained from the MD Anderson standardized data browser (http://bioinformatics.mdanderson.org/TCGA/databrowser/). RNA-Seq by Expectation-Maximization normalized expression values from the Illumina RNASeqV2 (genes) data sets were used.

Expression of mRNA signatures was calculated as the sum of z-score normalized expression of all mRNAs from the signature. Relapse-free survival (RFS) was estimated with the Kaplan–Meier method and compared with log-rank test (ggplot2 and survival R packages). Effects of molecular markers were estimated with the Cox proportional hazards model including univariate and multivariate analyses (survminer R package). Maximum sensitivity and specificity of logarithmic expression data were calculated using a receiver operator characteristic model (R package ggplot2, R version 4.0.2) to identify an optimized threshold value to discriminate high from low expression.

Gene set enrichment analyses (GSEA) were performed using the Hallmark data set with GSEA v4.1.0 (MSigDB v7.5, 1000 permutations; Broad Institute).[35] FDR values <0.25 were considered significant. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis[36] was conducted using the following settings: FDR stringency of 5%, minimum required interaction score: 0.15. Protein annotation through evolutionary relationship (PANTHER) database V.17.0 was used for additional gene ontology analyses.[37] All *P* values < .05 (2-sided) were regarded as significant.

Results

Study Design

This study aimed to elucidate mechanisms contributing to acquired FOLFIRI resistance and identify a gene expression signature potentially predicting FOLFIRI resistance in mCRC patients. The study design is presented in Figure 1. As a first step, FOLFIRI-resistant CRC cell lines were established and characterized according to cell morphology, gene expression of known epithelial-mesenchymal transition (EMT), and resistance markers and changes in the mutational status of cancer-associated genes. In the second step, comparative transcriptomic analyses of the parental and resistant cell lines were performed. Genes that were differentially expressed in at least 2 of the cell lines were identified and compared with expression data of the only clinical study we are aware of using a similar approach,[14] which, however, investigated primary resistance. Subsequently, a total of 14 identified single DEGs were tested for survival associations in 8 clinical CRC data sets. Five single gene expressions were significantly associated with RFS. In the last step, different expression combinations of the 5 genes were tested in univariate and multivariate Cox regression analyses. By this strategy, a FOLFIRI signature based on the combination of the expression levels of all 5 genes could be defined.

Establishment and Characterization of FOLFIRI-Resistant Colorectal Cancer Cell Lines

To establish a model system mimicking acquired FOLFIRI resistance in CRC patients, 3 CRC cell lines were continuously treated for about 12 months with increasing FOLFIRI concentrations and cell viability/IC_{50} was determined as described in section Materials and Methods. Three microsatellite stable CRC cell lines with activating alterations in the mitogen-activated protein kinase (MAPK) signaling pathway (Colo205, BRAF V600E; SW480, KRAS G12V; HT29, BRAF V600E) were used for this approach. Resistance indices (IC_{50} resistant/IC_{50} parental) achieved were: 29.42 in Colo205-R, 13.17 in SW480-R, and 2.43 in HT29-R cells (Fig. 1C and Supplementary Table S1). No morphologic changes were observed in HT29-R cells. However, in Colo205-R cells a switch to a mesenchymal phenotype was found in a large proportion of cells (Supplementary Fig. S1A, B). SW480-R cells, which usually grow as 2 subpopulations, namely E- (epithelial) and R- (round) types,[38] displayed enrichment of the R-type characterized by growth in tight clusters of piled-up cells (Supplementary Fig. S1C, D).

Since these changes are associated with EMT[39] and a more aggressive phenotype,[38] the expression of the EMT genes *CHD1* and *VIM* as well as the chemotherapy resistance related *TYMS* were initially investigated. A consistent significant *VIM* upregulation was observed in all 3 resistant derivatives (Colo205-R, 3.0-fold; SW480-R, 2.3-fold; and HT29-R, 4.8-fold; Fig. 1D). *CDH1* expression was not majorly changed in Colo205-R and HT29-R cells, whereas a significant increase was found in SW480-R cells (26.7-fold). *TYMS* expression was significantly enhanced in Colo205-R (4-fold) and HT29-R (1.63-fold). However, a 0.55-fold *TYMS* reduction was found in SW480-R cells.

To further investigate alterations during acquired FOLFIRI resistance due to genomic changes, the mutational and copy number status of 161 cancer-associated genes was investigated in parental and resistant cells by panel sequencing (Supplementary Table S2). The approach confirmed the known driver mutations in each cell line (Colo205, BRAF V600E; SW480, KRAS G12V; and HT29, BRAF V600E). However, only a single novel mutation was found in resistant derivatives. HT29-R cells displayed an Alpha Thalassemia/mental Retardation syndrome X-linked (*ATRX*) c.512G>T, p.(C171F) alteration that was not previously experimentally or clinically investigated. Prediction algorithms compiled at Varsome[24] evaluated this variant as likely pathogenic. Nevertheless, these results suggest that acquired FOLFIRI resistance is not driven by common DNA alterations in the investigated genes.

Taken together, we were able to establish 3 FOLFIRI-resistant CRC cell lines that display morphology and expression alterations suggesting partial EMT and *TYMS* upregulation in Colo205-R and HT29-R derivatives, whereas SW480-R seems to induce the epithelial marker *CDH1*, which is also reflected by the formation of tight cell clusters.

Identification of Differentially Expressed Genes and Signaling Pathways in FOLFIRI-Resistant Colorectal Cancer Cell Lines

In order to identify altered gene expression and mechanisms contributing to FOLFIRI resistance, a comparative transcriptomic analysis was performed. Since mRNA expression is influenced by cell culture conditions, parental and resistant cells of each cell line were seeded in 6 replicates and initial measurements of *CDH1*, *VIM*, and *TYMS* were done to select 4 samples each with similar expression levels of these genes (data not shown) for the following RNA sequencing. Despite this approach, outliers identified by principal component analysis based on transcriptomic expression data had to be removed from subsequent analyses. Four Colo205-P and 2 Colo205-R, 2 SW480-P and 4 SW480-R, as well as 3 HT29-P and 2 HT29-R expression data sets were utilized for further analyses. A principal component analysis of the leftover replicates shows clear transcriptome-wide differences in each parental cell line as well as its corresponding resistant derivative (Fig. 2A).

To elucidate these differences at the level of signaling pathways and cellular programs, GSEA was conducted using the HALLMARK gene sets. Gene sets significantly enriched in the resistant derivatives of each cell line included MAPK-associated (*KRAS_SIGNALING_UP*: Colo205 FDR = 0.001, SW480 FDR = 0.212, HT29 FDR = 0.017), immune response-related (*INFLAMMATORY_RESPONSE*: Colo205 FDR = 0.01, SW480 FDR = 0.142, HT29 FDR = 0.061), and EMT/cancer progression (*IL6_JAK_STAT3_SIGNALING*: Colo205 FDR = 0.001, SW480 FDR = 0.122, HT29 FDR = 0.105; *TNFA_SIGNALING_VIA NFKB*: Colo205 FDR = 0.001, SW480 FDR = 0.023, and HT29 FDR = 0.019) pathways (Fig. 2B).

On single gene level, 222, 30, and 47 DEGs ($log_2FC \geq 1$ and $P\text{-}_{adj} < 0.05$) were identified in Colo205-R, SW480-R, and HT29-R, respectively, of which 7 were shared between Colo205-R and HT29-R (*ANXA13*, *CAV2*, *CCL5*, *CD55*, *MMP7*, *PERP*, and *S100A4*), 2 between Colo205-R and SW480-R (*CEACAM6* and *FXYD3*), 1 between HT29-R and SW480-R (*KRT17*), and in addition 2 DEGs between all resistant cell lines (*GSN* and *TACSTD2*; Fig. 2C and Table 1). A gene ontology analysis using STRING based on the fold change of the 12 DEGs revealed significant enrichments of processes related to plasma membrane organization and cellular secretion (GO:0071944, *Cell periphery*, 11 genes, FDR = 0.0253; GO:0070062, *Extracellular exosome*, 7 genes, FDR = 0.0444; GO:0031982, *Vesicle*, 9 genes, FDR = 0.0444; GO:0005615, *Extracellular space*, 9 genes, FDR = 0.0253) as well as resistance to platinum-based chemotherapy (*PMID:37291676*, 4 genes, FDR = 0.0341; Fig. 2D). Corroborating results from the PANTHER database indicated a role of multiple genes in cellular adhesion/interaction (*CAV2*, *CEACAM6*, *GSN*, and *PERP*), calcium signaling and ion channel regulation (*ANXA13*, *FXYD3*, *S100A4*, and *TACSTD2*), as well as extracellular matrix degradation (*MMP7*).

In summary, FOLFIRI-resistant derivatives displayed activation of MAPK, immune response, and EMT pathways. Correspondingly, 12 DEGs significantly differentially expressed in at least 2 resistant cell lines were identified. These are associated with processes involved in tumor progression and chemotherapy resistance such as cellular secretion/exosomes, cell adhesion/interaction, calcium signaling, and EMT.[39-41]

Definition of a Five-Gene Signature Predicting Response to FOLFIRI Associated With Survival in Clinical Data Sets

To elucidate the potential role of the identified DEGs in a clinical setting, the Gene Expression Omnibus database was searched for clinical expression data sets addressing FOLFIRI resistance or response in mCRC. Only the GSE62322 data set comprised expression as well as clinical information of a study investigating response to FOLFIRI.[14] Data from 9 responders (R) and 11 nonresponders were available. The clinical data set contained expression data from 98 of the 284 in vitro identified DEGs. Of the 12 in vitro DEGs, shared between at least 2 cell lines, 3 were present but not significantly altered in nonresponders tumors (*GSN*, $P = .63$; *PERP*, $P = .99$; *TACSTD2*, $P = .11$). Two of the leftover

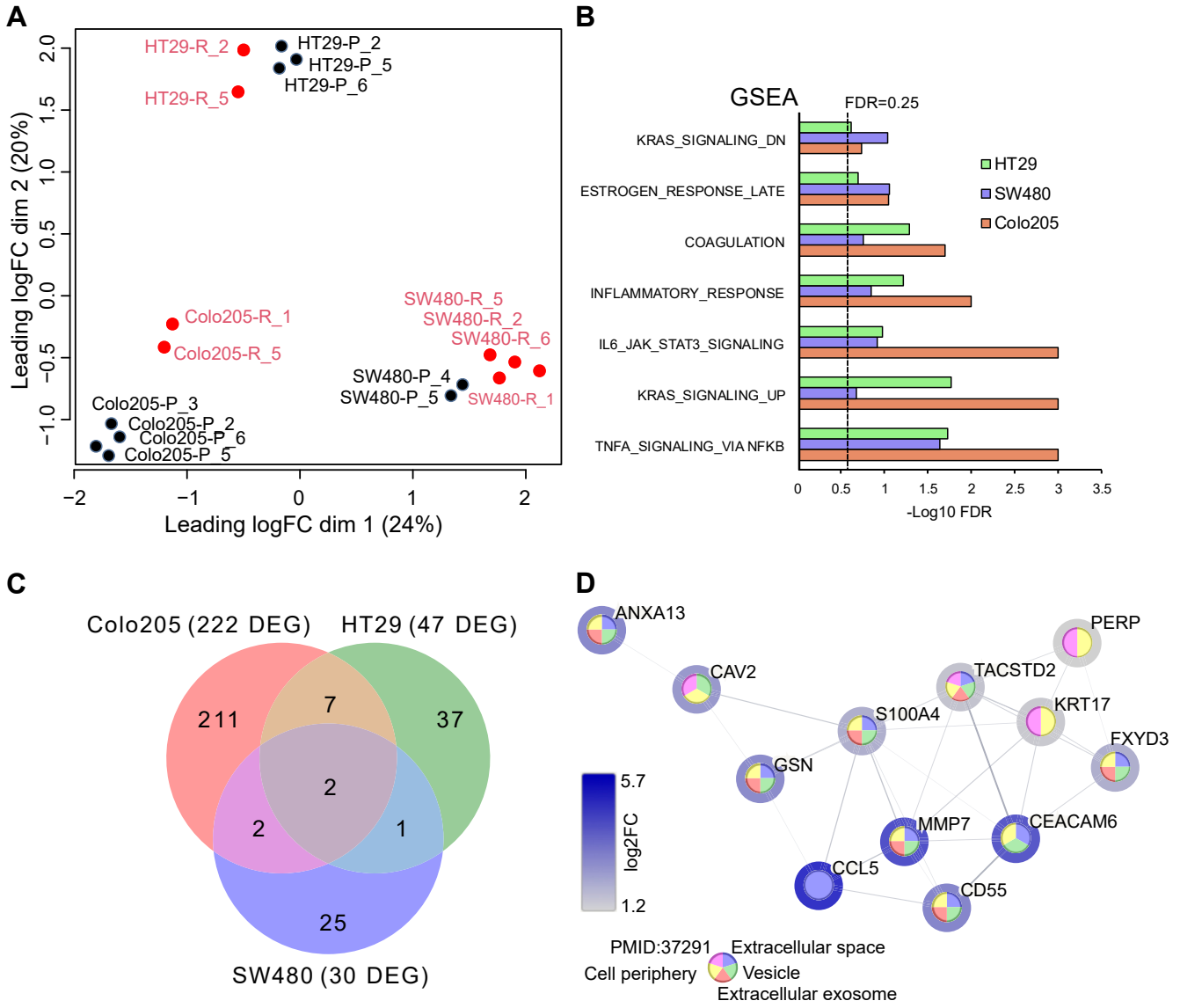

Figure 2.
Differential expression and gene ontology analyses of the indicated parental (P) and corresponding resistant (R) cell lines. (A) Principal component analysis after removal of outlier replicates used for subsequent expression analyses. (B) Gene set enrichment analysis (GSEA) using the HALLMARK gene sets. Pathways significantly enriched in all 3 resistant derivatives are depicted. (C) VENN diagram of differentially expressed genes (DEG) identified in resistant cell lines. (D) STRING analysis based on the fold change of the 12 identified signature genes in resistant cell derivatives. Known and predicted interactions as well as examples of significantly enriched pathways are shown. Line thickness represents confidence of protein-protein associations.

95 in vitro DEGs, namely *SERPINE2* and *TNC*, were also significantly differentially expressed ($log_2FC \geq 1$ and $P < .05$) in GSE62322 (Table 2).

In the next step, a clinical association of the 12 in vitro DEGs as well as *TNC* and *SERPINE2* was investigated by survival analysis using the CRC TCGA data set. Expressions of *CAV2* ($P = .000178$), *PERP* ($P = .00439$), *SERPINE2* ($P = .0292$), *TACSTD2* ($P = .000932$), and *TNC* ($P = .00584$) were associated with RFS (Fig. 3A-E). The results were confirmed for these 5 single genes in most of the 7 additional CRC patient cohorts (described in section Materials and Methods) and in the analysis of all samples in the 8 data sets (pooled). To identify a gene expression signature that might have an even stronger association with RFS than the single gene expressions, different combinations of the 5 gene expressions significantly associated with RFS were subjected to survival analyses. Combined expression of all 5 genes revealed the strongest negative correlation with RFS (TCGA, hazard ratio [HR] = 2.63, $P = 4.53 \times 10^{-6}$; pooled, HR = 1.85, $P = 6.44 \times 10^{-13}$, area under the curve [AUC] = 0.603), followed by a 2-gene signature comprised of *CAV2* and *TACSTD2* (TCGA, HR = 2.01, $P = 1.18 \times 10^{-3}$; pooled, HR = 1.64, $P = 5.92 \times 10^{-8}$, AUC = 0.58; Figs. 3F and 4; Supplementary Fig. S2). Because FOLFIRI is usually only applied to stage IV mCRC patients survival analyses were performed in stage IV samples pooled from all data sets (N = 260) using the 5-gene and 2-gene expression signatures (Fig. 4 D and Supplementary Fig. S2D). Confirming the stronger prognostic value, the correlation with RFS was more significant when utilizing the 5-gene signature (all cohorts stage IV only, HR = 2.46 vs 1.96, $P = 5.22 \times 10^{-9}$ vs 7.21×10^{-6}, AUC = 0.616 vs 0.6001).

Table 1
Differentially expressed genes in the FOLFIRI-resistant derivatives of at least 2 cell lines

Gene	Colo205		HT29		SW480	
	log_2FC	*P*-adj.	log_2FC	*P*-adj.	log_2FC	*P*-adj.
DEG in	Colo205 and HT29 and SW480					
GSN	3.22	8.38×10^{-9}	2.15	7.02×10^{-6}	2.51	8.78×10^{-4}
TACSTD2	1.62	1.47×10^{-2}	−1.86	3.51×10^{-2}	2.70	2.84×10^{-2}
DEG in	SW480 and Colo205					
CEACAM6	4.70	1.52×10^{-4}	1.71	ns	5.21	2.20×10^{-2}
FXYD3	2.19	1.07×10^{-5}	0.28	ns	2.88	1.75×10^{-5}
DEG in	Colo205 and HT29					
ANXA13	3.30	1.02×10^{-2}	5.52	2.95×10^{-4}	−0.28	ns
CAV2	2.82	1.45×10^{-2}	2.73	3.26×10^{-3}	1.19	ns
CCL5	5.79	9.28×10^{-4}	3.63	1.08×10^{-2}	−1.11	ns
CD55	3.38	1.44×10^{-2}	2.37	1.31×10^{-4}	2.32	ns
MMP7	4.91	2.43×10^{-8}	1.52	1.72×10^{-2}	0.22	ns
PERP	1.27	9.23×10^{-4}	1.51	1.11×10^{-3}	0.96	ns
S100A4	2.19	2.15×10^{-12}	−2.06	2.93×10^{-12}	0.23	ns
DEG in	SW480 and HT29					
KRT17	1.44	ns	3.00	1.30×10^{-2}	3.38	2.00×10^{-2}

FC, fold change; ns, not significant; *P*-adj., *P*-adjusted.

Moreover, univariate and multivariate Cox regression analyses were performed using the TCGA data set and the following factors: 5-gene and 2-gene signatures, microsatellite instability (MSI) (MSI vs MSS), pT (T3+T4 vs T1+T2), pN (pN1+pN2 vs pN0), and pM (pM1 vs pM0; Table 3). The univariate analysis revealed a highly significant association of the 5-gene signature (HR = 2.63, $P = 9.7 \times 10^{-6}$) and, as expected, pN (HR = 2.57, $P = .42 \times 10^{-7}$) and pM (HR = 4.16, $P = 9.1 \times 10^{-11}$) status, whereas a lower significance was observed for the 2-gene signature (HR = 2.01, $P = .0015$) and pT status (HR = 2.69, $P = .0018$). The multivariate analysis showed that only the 5-gene signature (HR = 1.89, $P = .0202$) and, again anticipated, pM status (HR = 3.01, $P = 5.6 \times 10^{-5}$) are independent factors affecting the prognosis of CRC. No significant association was found for the 2-gene signature (HR = 1.48, $P = .1399$).

Taken together, by utilization of a cell culture model mimicking acquired FOLFIRI resistance and subsequent data reconciliation in clinical data sets, we have identified a 5-gene signature that could be useful to predict resistance to FOLFIRI treatment in mCRC.

Discussion

In mCRC, recommended regimens for first-line chemotherapy therapy are FOLFIRI, FOLFOX, or FOLFOXIRI.[5] Apart from clinical parameters (eg, physical condition), there are no specific molecular markers, which serve as a decision tool when choosing between the 3 regimens. However, therapy resistance is a major hindrance in curative treatment. In this respect, the identification of new biomarkers could assist in choosing the most suitable first-line regimen as well as in providing a tool to predict therapy response/resistance and survival.

Here, we aimed to elucidate molecular mechanisms of acquired FOLFIRI resistance in CRC and to identify novel biomarkers and gene expression signatures associated with secondary FOLFIRI resistance. We present a 5-gene signature composed of *CAV2*, *PERP, SERPINE2*, *TACSTD2*, and *TNC* that is associated with survival in mCRC and may serve to predict FOLFIRI resistance in a clinical setting in the future. Moreover, each member of the signature might represent an addressable therapeutic target. In addition, gene ontology analyses supported the involvement of EMT, MAPK, and immune response-associated pathways in acquired FOLFIRI resistance.

To mimic acquired FOLFIRI resistance, 3 CRC cell lines were continuously treated with increasing FOLFIRI doses. Only a single mutation (C171F, predicted as likely pathogenic) in the ATRX tumor-suppressor gene[42] was observed in 1 resistant cell line that was not previously associated with FOLFIRI resistance either experimentally or clinically. No other additional DNA alterations were found in the investigated 161 cancer-associated genes in the resistant derivatives. These results are supported by earlier findings, suggesting that secondary chemotherapy resistance of mCRC is not primarily mediated by novel mutations in cancer genes[7] and that metastatic genomes are not significantly different from primary tumor genomes.[43] These and other studies rather proposed expression changes as important drivers of acquired chemoresistance, tumor progression, and metastasis.[7,8,14]

Table 2
Expression of the 5-gene FOLFIRI resistance signature in resistant cell lines and the GSE62322 data set

Gene	Colo205		HT29		SW480		GSE62322		
	log_2FC	*P*-adj.	log_2FC	*P*-adj.	log_2FC	*P*-adj.	log_2FC	*P*	*P*-adj.
TACSTD2	−1.86	.035	1.62	.015	2.70	.028	−0.46	.11	.67
CAV2	2.73	3.26×10^{-3}	2.82	.015	1.19	1.0	na	na	na
PERP	1.51	1.11×10^{-3}	1.27	9.23×10^{-4}	0.96	1.0	−0.003	.99	.99
TNC	na	na	3.62	.04	4.77	.36	−1.61	.012	.39
SERPINE2	−3.46	.40	6.99	1.38×10^{-5}	2.07	1.00	−1.14	.027	.49

FC, fold change; na, not available; *P*, *P* value. *P*-adj., *P*-adjusted.

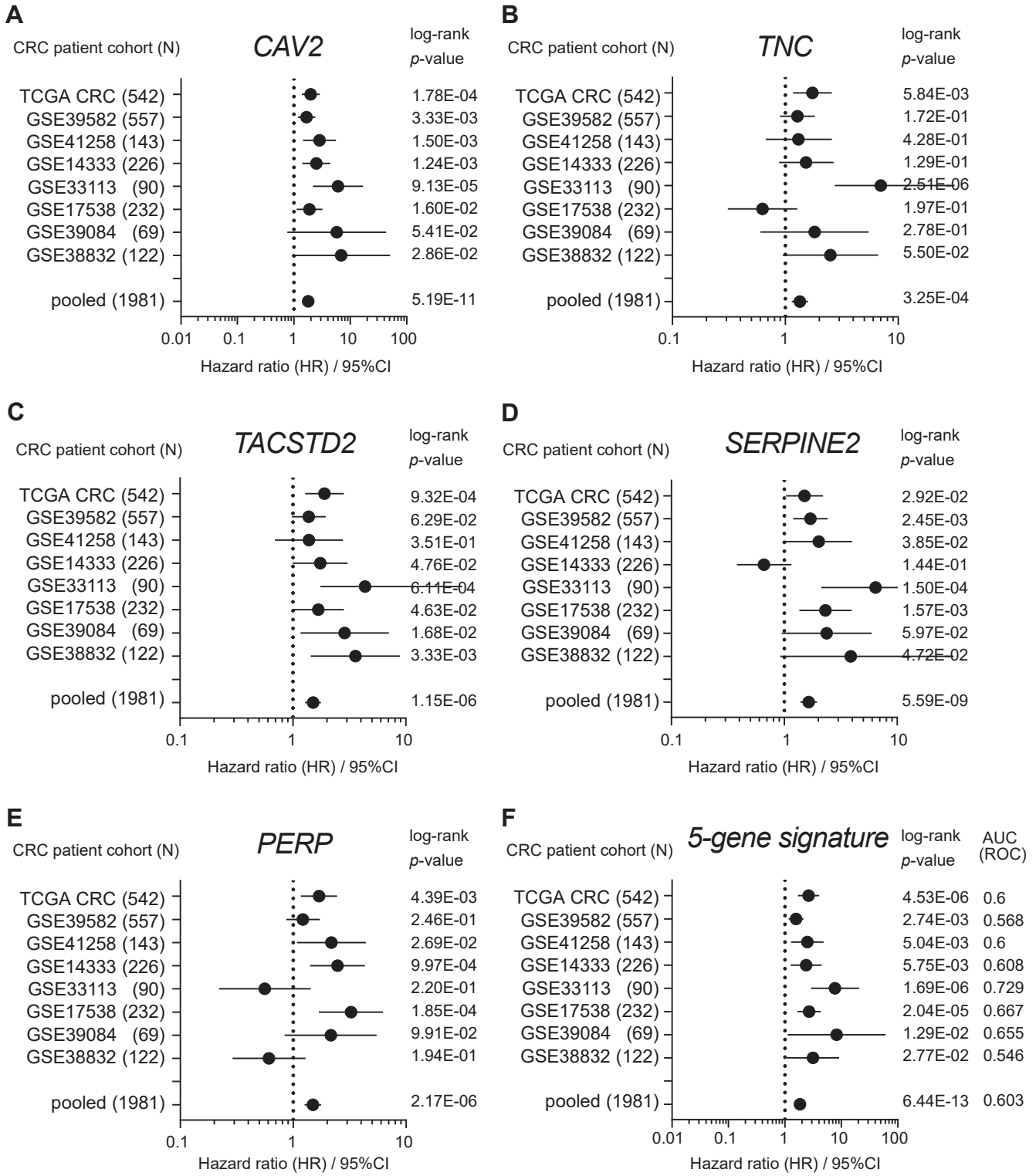

Figure 3.
Survival analysis (relapse-free survival [RFS]) of CRC patients stratified by low and high expression of the identified single DEG (A-E) and the 5-DEG signature (F) in 8 clinical CRC data sets. Hazard ratios (HR) including 95% confidence intervals (CI), *P* values (log-rank test), and patient numbers for each clinical data set and all samples (pooled) are indicated. AUC, area under the curve. ROC, receiver operating characteristics.

Based on this, a transcriptomic analysis was performed (1) to identify differentially activated pathways, (2) to identify DEGs, and (3) to establish an expression signature associated with FOLFIRI resistance and survival.

First, GSEA revealed enrichment of EMT, MAPK, and immune response-associated pathways in all 3 FOLFIRI-resistant derivatives. EMT is required for the invasion of tumor cells into the surrounding matrix and the formation of distant metastasis.[3,39,44]

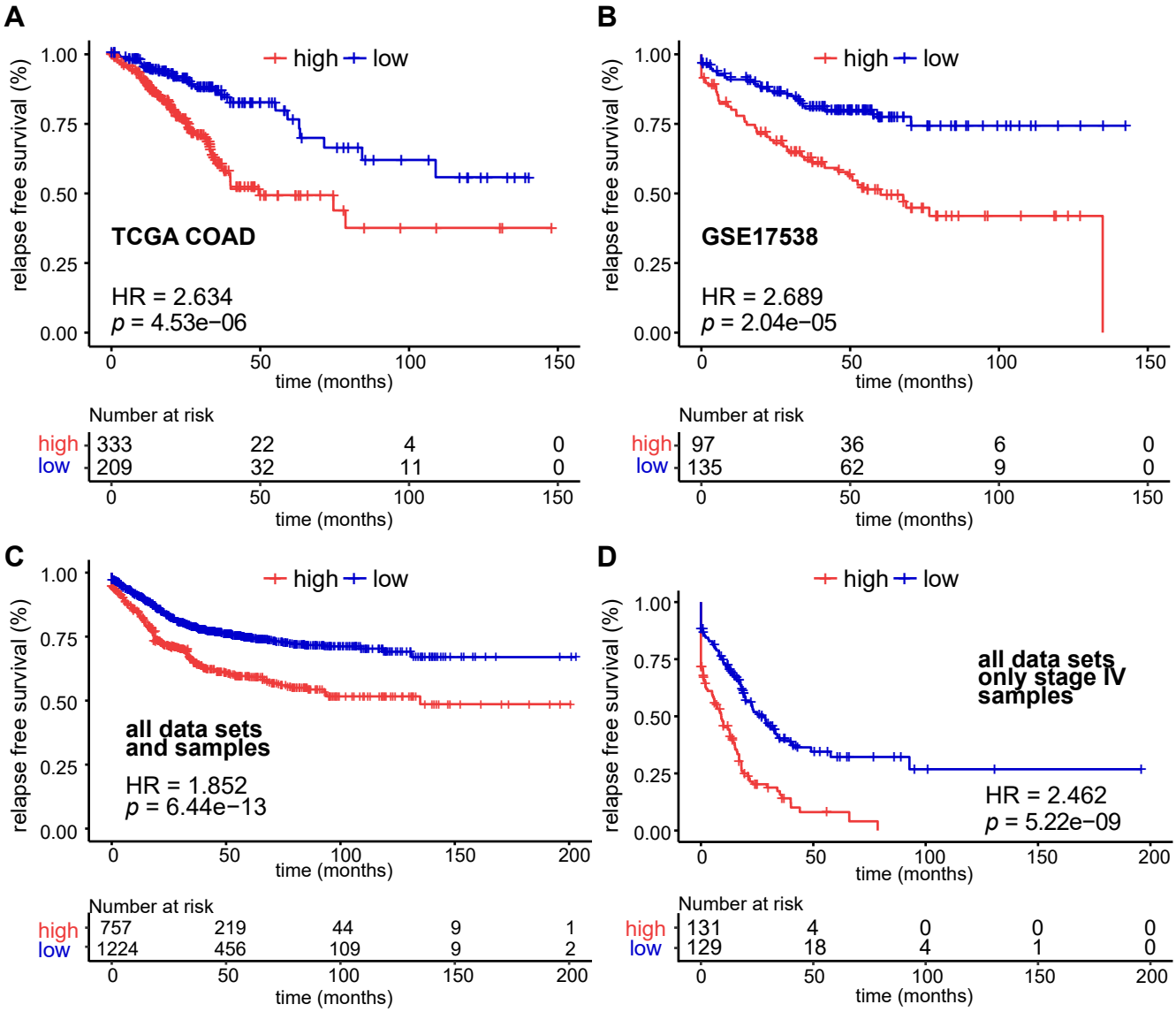

Figure 4.
Survival analysis (RFS) of CRC patients stratified by low and high expression of the identified 5-DEG signature. Kaplan-Meyer estimates using the TCGA COAD (A), GSE17538 (B), and all 8 CRC data sets (C, all stages; D, stage IV only) are presented. Hazard ratios (HR), *P* values (log-rank test), and patient numbers are indicated. COAD, colorectal adenocarcinoma; CRC, colorectal cancer; DEG, differentially expressed gene; RFS, relapse-free survival; TCGA, the cancer genome atlas.

Various studies demonstrated that cells that have undergone EMT acquire resistance to several targeted drugs and chemotherapeutic treatments in various cancers, including CRC.[39,44] However, an association between FOLFIRI treatment and EMT in CRC was only observed in a few studies. In line with our findings, Napolitano et al[45] described induction of the EMT program after FOLFIRI treatment in a patient-derived tumor xenograft mouse model using BRAF V600E positive mCRC tumors. In addition, we and Napolitano et al[45] observed an activation of the MAPK pathway in the FOLFIRI-resistant derivatives or treated xenografts,

Table 3
Univariate and multivariate Cox regression analyses results of the TCGA COAD (N = 542) cohort

TCGA COAD	Univariate analysis				Multivariate analysis			
Variable	HR	CI lower	CI upper	*P*	HR	CI lower	CI upper	*P*
5-gene signature (high vs low)	2.63	1.71	4.04	9.7×10^{-6}	1.89	1.10	3.23	.0202
2-gene signature (high vs low)	2.01	1.31	3.09	.0015	1.48	0.88	2.50	.1399
MSI (MSI vs MSS)	0.82	0.46	1.47	.5114	1.24	0.65	2.34	.5140
pT (T3+T4 vs T1+T2)	2.69	1.45	5.01	.0018	1.62	0.78	3.35	.1932
pN (pN1+pN2 vs pN0)	2.57	1.78	3.71	4.2×10^{-7}	1.35	0.81	2.23	.2464
pM (pM1 vs pM0)	4.16	2.70	6.40	9.1×10^{-11}	3.01	1.76	5.15	5.6×10^{-5}

95% CI, 95% confidence interval; COAD, colorectal adenocarcinoma; HR, hazard ratio; *P*, *P* value; TCGA, the cancer genome atlas.

respectively. This result is somehow surprising as the parental cells or tumors carry activating mutations in the key MAPK pathway genes *BRAF* or *KRAS*. This finding might be explained by cross-talk of the MAPK, PI3K, and EMT/b-Catenin pathways leading to an even stronger MAPK activation.[46,47] Activation of MAPK signaling is related to resistance to chemotherapy, including irinotecan,[19,47] further supporting our results.

Thus, our results further emphasize the importance of EMT and MAPK pathways in FOLFIRI resistance and confirm the plausibility of the data set generated with our model system.

Second, comparative transcriptomic analyses revealed 12 genes that were significantly differentially expressed in at least 2 of 3 FOLFIRI-resistant CRC cell derivatives. Gene ontology analyses showed a functional relation of the 12 genes to processes involved in plasma membrane organization and cellular secretion as well as resistance to platinum-based chemotherapy. Moreover, multiple of the 12 genes play roles in cellular adhesion/interaction, calcium signaling, and ion channel regulation as well as extracellular matrix degradation. These processes are well known to drive EMT, tumor progression, and chemotherapy resistance[39-41] and are in line with the whole transcriptome pathway results.

Third, we aimed to define a gene signature associated with FOLFIRI resistance. Therefore, our data set was compared with other available data sets related to FOLFIRI resistance in CRC. Only a single data set, namely GSE62322, comprised both expression data and clinical information investigating the response to FOLFIRI.[14] However, this study analyzed expression data of therapy-naive primary tumors from 20 patients prior to first-line FOLFIRI treatment reflecting primary/intrinsic resistance, whereas our cell culture approach mimicked acquired resistance. This may explain why only 2 genes, *SERPINE* and *TNC*, were found significantly differentially expressed in both data sets. In the next step, the prognostic potential of the 12 DEGs as well as *SERPINE* and *TNC* was analyzed utilizing the CRC TCGA data set and validated in 7 additional CRC patient cohorts. Expressions of 5 single genes, *CAV2*, *PERP*, *SERPINE2*, *TACSTD2*, and *TNC* were significantly associated with RFS. Importantly, the combined expression signature of these 5 genes revealed an even stronger negative correlation with RFS. In this respect, the 5-gene signature may have prognostic value and aid in predicting FOLFIRI resistance in a clinical setting.

Finally, each of the signature genes may represent a potential individual therapeutic target or prognostic factor, and thus, the role of each gene is discussed.

Caveolin-2 (*CAV2*) is a member of the caveolin family constituting the main integral proteins of non-planar lipid rafts called caveolae.[48] Caveolae are mechanosensitive structures that act as signal transducing platforms controlling various cellular programs and deregulation of their components are implicated in tumor progression and metastasis.[48,49] Little is known about the role of *CAV2* in driving cancer progression or treatment resistance. *CAV2* was upregulated in Adriamycin-resistant MCF-7 breast cancer cells, and its expression was associated with increased caveolae formation.[50] The authors and subsequent studies proposed an involvement of calveolins in drug efflux.[50,51] In addition, *CAV2* is part of a 7-gene prognostic CRC signature from CRC cell lines pre- and post-5−FU treatment.[52] In lung adenocarcinoma and esophageal squamous carcinoma patients, *CAV2* is part of an expression signature associated with cisplatin sensitivity.[53]

The second gene, *PERP*, is a member of this cisplatin signature as well.[53] P53 Apoptosis Effector Related To PMP22 (*PERP*) is a plasma membrane protein involved in desmosomal cell-cell adhesion, driving EMT as well as p53-mediated apoptosis.[54,55] Its role in cancer progression and therapy resistance is controversial, as its expression is linked to cisplatin sensitivity,[53] and *PERP* was ascribed a role as a tumor-suppressor gene in several studies.[55,56] Thus, the observed *PERP* upregulation in 2 of our FOLFIRI-resistant derivatives and the association of high *PERP* expression with reduced RFS in clinical CRC data sets is quite surprising, especially considering that *PERP* is a direct transcriptional target of p53[57] and all 3 cell lines used in our study, as well as 60% to 70% of CRC patients, carry a *TP53* mutation.[7] However, *PERP* is also a transcriptional target of the EMT transcription factor SNAI1 and involved in mediating early EMT,[58] which can foster treatment resistance[39,44] and is reflected in our pathway analysis by EMT enrichment.

The third gene of our FOLFIRI resistance signature is *Serpin Family E Member 2* (*SERPINE2/PN-1*), which is an extracellular serine protease inhibitor targeting, for example, thrombin and urokinase that contributes to cancer progression mainly by tumor matrix remodeling.[59-61] In line with our results, significantly higher *SERPINE2* expression levels were observed in a CRC patient cohort compared with normal tissue.[62] *SERPINE2* is also part of an expression signature associated with response to FOLFIRI (primary resistance).[14] Interestingly, *SERPINE2* expression is driven by MAPK/extracellular signal regulated kinase signaling and upregulated in CRC cell lines exhibiting *KRAS* or *BRAF* mutations.[63] As discussed above, it is rather surprising to find an increased expression in the already KRAS/BRAF-mutated FOLFIRI-resistant derivatives. This might be explained by recent studies that identified a positive feedback loop where SERPINE2 activity leads to the induction of its own expression via EGF/PKC/MAPK/EGR1 signaling.[64] Thus, SERPINE2 expression might be a prognostic marker for the application of MAPK/extracellular signal regulated kinase inhibitors such as trametinib.

The fourth member *Tumor-Associated Calcium Signal Transducer 2* (*TACSTD2*) encodes for the transmembrane glycoprotein Trophoblast cell surface antigen 2 (Trop-2). Trop-2 transduces calcium signals and is overexpressed in various solid tumors.[65] Overexpression in CRC correlates with poor survival rates and increased metastasis.[66,67] Trop-2 mediates loss of cell-cell-adhesion and dysregulation of the β-Catenin pathway[66] as well as activation of the MAPK-signaling pathway.[65] These results are in line with our pathway and survival analyses. Current targeted therapeutic strategies rather utilize Trop-2 as an anchor to direct cytotoxic agents to tumor cells to induce apoptosis.[68] Therefore, antibody-drug conjugates (ADCs) consisting of a Trop-2-specific antibody conjugated to topoisomerase inhibitors are used. Two examples are: (1) Sacituzumab govitecan (cytotoxin: SN-38, active metabolite of irinotecan), which is approved for the treatment of certain subtypes of metastatic breast cancer and metastatic urothelial cancer.[68] The results from the phase I/II IMMU-132-01 basket trial indicated a poor objective response rate of 3.2% in the CRC subcohort.[69] However, patients were enrolled regardless of their Trop-2 expression level, and 94% of the CRC patients were pretreated with a regime containing irinotecan, which might have led to an acquired resistance. Thus, a Trop-2 ADC carrying a different chemotherapeutic agent might result in a much better objective response rate in FOLFIRI pretreated patients. (2) The anti-Trop-2 ADC example, namely datopotamab deruxtecan (Dato-DXd), utilizes a derivative of exatecan, which is also a topoisomerase I inhibitor with a 10-fold higher inhibitory potency than SN-38.[70] Dato-DXd is nearing approval for treatment of breast[71] and lung cancers.[72] Currently, the efficacy of Dato-DXd as monotherapy and in combination with other anticancer agents is tested in the phase 2 trial TROPION-PanTumor03 in CRC patients prospectively selected for TROP2 expression.[73]

The fifth FOLFIRI resistance signature molecule Tenascin-C (*TNC*) is a large, multimodular, extracellular matrix glycoprotein

exerting diverse functions.[74] It is expressed in various cancers including CRC and secreted by tumor and stroma cells that contribute to cancer progression and metastasis.[74,75] *TNC* was defined as a potential prognostic factor in aflibercept plus FOLFIRI-treated mCRC patients,[76] thereby supporting our findings associating *TNC* with FOLFIRI resistance. In addition, *TNC* plays an important role in CRC progression and metastasis[75,77] and was also considered a diagnostic blood marker in CRC patients.[78] Moreover, *TNC* was upregulated in therapy-naive CRC, and *TNC* was an independent predictor of poor overall survival and disease free survival.[77] Corresponding with our pathway analyses, *TNC* expression correlated with EMT.[75,77,79] Due to its role in various cancer types, multiple strategies targeting TNC comprising, for example, ADCs and Nanobodies, were developed and are currently tested in preclinical and clinical studies.[80]

The presented study has limitations. (1) Our in vitro cell culture system of acquired FOLFIRI resistance does not necessarily reflect the resistance mechanisms in CRC patients. Nevertheless, it is indisputable that much of the knowledge on molecular cancer biology and treatment resistance mechanisms is based on initial in vitro and preclinical animal models that represent neither the physiological conditions nor the complex microenvironment of human tumors. Moreover, many findings from resistance cell culture models could previously be translated into preclinical and clinical studies, leading to better stratification of patients regarding treatment options. In addition, our results are plausible because we identified mechanisms, such as enriched EMT and MAPK signaling, and genes that were previously associated with resistance mechanisms in the very few available FOLFIRI studies/data sets. (2) No clinical validation cohort and/or transcriptomic data as well as response data were publicly available reflecting our approach of secondary/acquired FOLFIRI resistance, as most studies investigated expression data in primary tumors, and thus, associated FOLFIRI responses resemble primary/intrinsic resistance. Therefore, to confirm the importance of the 5-gene signature and the single signature genes in CRC, we utilized common CRC data sets in our survival analyses. (3) These survival analyses were not stratified for patients treated with a FOLFIRI regime, as much of the data on the used treatment regime were unavailable, or the resulting patient numbers in each subgroup were too low or unequal to perform statistical analyses. Nevertheless, we believe that this work contributes to the limited number of studies to date addressing molecular changes in secondary/acquired FOLFIRI resistance in CRC.

In conclusion, we describe a 5-gene FOLFIRI resistance CRC signature, composed of *CAV2*, *PERP*, *SERPINE2*, *TACSTD2*, and *TNC*, associated with RFS, which might be useful in future clinical practice to predict FOLFIRI resistance, thereby avoiding unnecessary ineffective treatment. Importantly, the role of the signature genes as potential drug targets was emphasized in many previous studies. Nevertheless, the 5-gene signature needs to be tested in further studies.

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Author Contributions

Conceptualization, A.J. and J.K.; Data curation, C.P., E.P. and J.K.; Formal analysis, C.P., E.P., L.T., M.R., P.L., and J.K.; Investigation, C.P., E.P., L.T., P.L., M.R., H.H., J.W., F.K., J.N., A.J., and J.K.; Project administration, A.J. and J.K.; Resources, H.H., J.W., F.K., J.N., J.K., and A.J.; Supervision, A.J. and J.K.; Validation, C.P., E.P., M.R., and J.K.; Visualization, J.K.; Writing—original draft, C.P., E.P., and J.K.; Writing—review and editing, all authors. All authors reviewed the final version and approved the submission.

Data Availability

Sequencing data presented in this study are available on request from the corresponding author.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Ethics Approval and Consent to Participate

This study did not involve any animal experiments or the use of human subjects.

Supplementary Material

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